'EVALUATION OF THREE GENUS SPECIFIC SEROLOGICAL TESTS FOR THE DIAGNOSIS OF LEPTOSPIROSIS'

BY

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DISSERTATION SUBMITTED TO

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION & RESEARCH

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UNDER THE GUIDANCE OF

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LIST OF ABBREVIATIONS

IgM	-	Immunoglobulin M
IgG	-	Immunoglobulin G
ELISA	-	Enzyme Linked Immunosorbent Assay
IIFA	-	Indirect Immunofluorescent Antibody technique
MAT	-	Microscopic Agglutination Test
FITC	-	Fluorescein IsoThiocyanate Conjugate
WHO	-	World Health Organization
ICU	-	Intensive Care Unit
CDC	-	Centre for Disease Control and prevention
BSA	-	Bovine serum albumin
EMJH	-	Ellinghausen-McCullough-Johnson-Harris
TPB	-	Tryptone Phosphate Broth
CSF	-	Cerebro Spinal Fluid
HIV	-	Human Immunodeficiency Virus
DNA	-	Deoxyribo Nucleic Acid
rRNA	-	Ribosomal Ribo Nucleic Acid
PCR	-	Polymerase Chain Reaction
OMP	-	Outer Membrane Proteins
LPS	-	Lipopolysaccharide
DFM	-	Dark Field Microscopy
HLA	-	Human Leucocyte Antigen
PBS	-	Phosphate Buffered Saline
OD units	-	Optical Density units
GMT	-	Geometric Mean Titre

ABSTRACT

INTRODUCTION:

Leptospirosis, an acute febrile illness caused by *Leptospira interrogans*, is a widespread zoonotic disease. It is endemic in many parts of Karnataka including Kolar region. Due to its protean clinical manifestations and difficulty in isolation of the organism, diagnosis of leptospirosis is difficult. Early diagnosis and treatment is important, as untreated cases may progress rapidly and mortality rates are high in severe cases. Serological tests based on detection of antibodies remain the most practical methods of diagnosis. It is often necessary to use multiple tests to make a reliable diagnosis as cases may be missed when a single test is used. Commercially available IgM ELISA is used in many laboratories for the diagnosis of recent infection. But it has been reported to give false positive results in other infections like Dengue, Malaria and Typhoid. IIFA has been reported to be an alternative sensitive and specific test for the initial diagnosis of leptospirosis. To make the diagnosis of leptospirosis more specific and useful in patient care, we planned to evaluate two screening tests, I_gM ELISA and IIFA, and to further evaluate the positive results obtained in these two tests by MAT. However, as Fluorescein isothiocyanate (FITC) conjugated antihuman IgM is difficult to procure, we used antihuman IgG FITC conjugate in IIFA. Hence we conducted the study, to evaluate IgM ELISA and IIFA as screening tests and positives detected by these tests were evaluated by MAT.

MATERIALS AND METHODS:

Serum samples collected during the acute phase of illness from 2362 patients, who visited R.L.Jalappa Hospital with fever and were clinically suspected of

leptospirosis, were screened for evidence of IgM antibodies to *Leptospira* by a commercially available ELISA kit. A subset of 250 serum samples was screened by IIFA (using Patoc strain of leptospira and antihuman IgG FITC conjugate. Patoc strain is a saprophytic strain of *Leptospira* which is genus specific) and positives detected by either of these two tests were subjected to MAT (using Patoc strain of *Leptospira*) for confirmation. Paired samples available in fifteen patients who were positive for IgM class of antibodies were subjected to both IIFA and MAT parallelly.

RESULTS:

Among 2362 serum samples from patients who were suspected of leptospirosis and screened by IgM ELISA, Seropositivity rate was found to be 3.3%. Majority of the IgM ELISA positive patients were adults belonging to 3rd and 4th decades of life accounting for 51.28% of the patients. Preponderance of women is seen in our study who accounted for 65% of the patients. Most of the cases were seen during January and February 2012 accounting for 55% of the patients.

IIFA could detect only 68% of the cases that were positive by IgM ELISA and in addition, it detected 2% of the cases that were negative by ELISA. However, the concordance between the two tests was found to be 92%.

MAT confirmed 88% of the cases that were positive by IgM ELISA, thereby accounting for a false positivity rate of 12% in IgM ELISA. Whereas, MAT confirmed only 81.58% of the cases that were positive by IIFA.

The congruity between all the three tests was found to be 55.56%. Evaluating the positive result in any two of the three serological tests, for a case to be considered as a serologically presumptive case of leptospirosis, 88.88% were found to be

presumptive cases of leptospirosis.

Among 15 paired (Acute-convalescent) samples, MAT demonstrated fourfold rise in titres in 66.66% of the cases which is serologically diagnostic of Leptospirosis, whereas IIFA did so only in 13.33% of the cases.

CONCLUSION:

Our study showed that IgM ELISA can be considered as a good screening test. IIFA did not show any additional advantage for the diagnosis of leptospirosis. However, further study on IIFA using anti human IgM FITC conjugate is necessary for proper evaluation. MAT is an ideal test to demonstrate four-fold rise in antibody titres in paired samples and IIFA does not seem to be an ideal test for paired samples.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF CHARTS

1. INTRODUCTION

Leptospirosis is an acute febrile illness caused by pathogenic strains of *Leptospira* belonging to the species *Leptospira interrogans*. It is a widespread Zoonotic disease and is a common cause of acute febrile illness throughout the wet tropical regions of the world.[1, 2] Studies show that leptospirosis is endemic in many parts of Karnataka and epidemics are seen during rainy seasons. [4, 5, 6] Leptospirosis is also endemic in Kolar district of Karnataka as evidenced by cases diagnosed at R.L.Jalappa Hospital, Kolar.

Clinical diagnosis of leptospirosis is difficult due to its varied clinical manifestations ranging from mild flu like illness to fatal hepatorenal syndrome, pulmonary haemorrhage, myocarditis and meningitis. Also, the signs and symptoms resemble a wide range of bacterial and viral diseases due to which the diagnosis is often missed.[1, 2, 3] If not treated in time, the illness can progress rapidly and mortality rates are high in severe cases. It is therefore important to differentiate leptospirosis from other acute febrile illnesses and to diagnose early so that prompt specific treatment can be instituted. [2]

Isolation of *Leptospira* from blood, urine, other body fluids and tissues constitutes the definitive diagnostic test. However, the organism takes 4-6 wks to grow and growth is unreliable, thus making culture unsuitable for routine diagnosis in patients requiring medical care. So, the serological tests remain the most practical methods of diagnosis. It is often necessary to use multiple tests to make a reliable diagnosis, as cases may be missed when we use a single test.[1, 3, 7] Usually, the serological diagnosis of leptospirosis rests on Microscopic agglutination test (MAT), Indirect Immunofluorescence antibody assay (IIFA), IgM Enzyme linked

immunosorbent assay (ELISA), Latex agglutination or Immunochromatographic tests.[7]

Among these tests, MAT has been considered the reference standard test. However, it is not useful for early diagnosis of leptospirosis and is also cumbersome to perform. In this situation, I_gM ELISA and the related Immunochromatographic tests have been used as diagnostic tests for leptospirosis. [7, 8] But these tests have the limitation of giving the positive results in other infections like Dengue, Malaria and Typhoid. [7, 8, 9] IIFA has been reported to be an alternative sensitive and specific test for the initial diagnosis of leptospirosis. [10]

To make the diagnosis of leptospirosis more specific and useful in patient care, we planned to evaluate the results of two screening tests, I_gM ELISA and IIFA, and if positives detected by these two tests taken together concord with MAT test, the need for performing MAT test can be obviated. If these two tests together are found suitable, they can be employed to make an accurate diagnosis replacing MAT test.

2. OBJECTIVES

1. To screen the acute sera of the patients suspected of leptospirosis by I_gM ELISA.
2. To screen Acute-convalescent pairs of sera (as per availability) or a single serum Sample by IFA using Patoc strain of *Leptospira* and to titrate the end point in positive cases.
3. To correlate the results of above tests with MAT using Patoc strain of *Leptospira*, on acute-convalescent pairs of sera (as per availability).

3. REVIEW OF LITERATURE

3. A: HISTORY

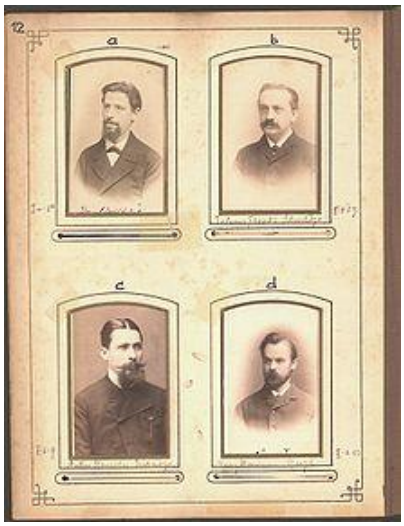
Leptospirosis is probably a disease of antiquity and an epidemic disease of native population. Epidemics of jaundice with characteristics of leptospirosis can be traced back to earlier centuries. The descriptions of this disease are seen in ancient Chinese and Japanese literature which describes that leptospirosis was an occupational hazard of rice harvesting population in these countries. [1, 2] The names Akiyama fever and Autumn fever is derived from these ancient records. It was also described in few European countries as the disease syndrome occurring in sewer workers. [2]

Jaundice with fever has also been described in ancient Indian literature but the cause of the illness was not clearly known. [11] Leptospirosis is postulated as the cause of epidemic among native Americans along the coast of present day Massachusetts in 1620, during which the disease killed most of the native population.[12] Transmission during this epidemic is thought to be due to Rodent reservoirs from the European ships, infecting and contaminating land and fresh water and exposure of the local population to these sources due to local ecology and high risk practices of the population. [12] It is the same disease from which Napolean's army suffered during Egyptian campaign as well as, the troops in American civil war. [13]

Leptospirosis as a disease entity was probably described almost simultaneously by three different physicians in 19th century, which is the period when most of the microbial causative agents were discovered and also many scientific discoveries were made. [3,12] However, the honour of first describing the characteristic icteric leptospirosis goes to Adolf Weil, a German physician, who in 1886, published his

description regarding a case of an acute febrile illness with jaundice associated with enlargement of spleen and nephritis.[1,2,3] Even today, his name has been celebrated as an eponym for icteric leptospirosis.

Figure 1: Adolf Weil (1848 – 1916)



University of Heidelberg tableau- Adolf Weil at upper left

(Courtesy: Bibliography @ who named it)

Adolf Weil was born in 1848 at Heidelberg. He pursued his Medicine at university of Heidelberg and in 1886, was appointed as a professor of special pathology and therapy at the University of Dorpat. During the course of his work, he described the disease in a farmer who presented with fever, muscular pain, and jaundice. He was found to have hepatosplenomegaly and nephritis. He wanted to further investigate and workout on the disease but unfortunately, he contracted tuberculosis of larynx and permanently lost his voice. He continued to suffer from tuberculosis and finally died in 1916. [14]

Similar descriptions were made by Landouzy in 1883 and Vasiliev in 1888, but as it was not published, it came to be known later. [2, 3, 13]

Investigations on yellow fever led to the discovery of *Leptospira*. The organism was discovered in the tissues of a patient who was suspected to have died of yellow fever. During the early part of 20th century, yellow fever was prevalent in most parts of the world. Research was on its way to find out the causative agent of yellow fever. [3] The spirochaetes as the causative agents of leptospirosis was suggested by Schaudinn and Novy. It was Stimson, in 1907, who first observed and demonstrated these spirochaetes. Stimson was an assistant surgeon in public health and marine hospital service in San-Fransisco, United states. While investigating on yellow fever, he collected tissues like liver, kidneys, heart and brain from patients who were suspected to have died of yellow fever. He planned to do levaditi's staining on these tissues but most of these tissues were fixed in solutions other than formalin on which, this method of staining could not be performed. Fortunately tissues from one patient were fixed in formalin and these tissues were subjected to levaditi's staining. In the tubules of kidney, he observed a very definitive organism which was opaque black and the appearance which strongly resembled a spirochaete with regular series of alternate curves and one or both the extremeties often bent in the form of a hook. He named it *Spirocheta interrogans*, as it resembled a question mark. [15]

In 1914, a saprophytic spirochaete, later named *Leptospira biflexa* was described but was not cultured. [2, 3]

The scene shifts to Japan, which was known to be endemic and prone to epidemics of Weil's disease. In 1915, Inada, Ido and Ito studied the aetiology and mode of infection in Leptospirosis by animal experiments. In their experiments and the course of investigation, they discovered a spirochaetal microorganism by injecting blood from the patients with features of weil's disease and observing and culturing

them from various experimental animals like rabbits, rats and guinea pigs. They announced the discovery of *Leptospira*. [16]

Almost simultaneously at this time, two groups of German physicians, Uhlenhuth and Fromme and Hubener and Reiter, while investigating the aetiology of fever in German soldiers afflicted by "French disease" in the trenches of northeast France, detected spirochaetes in the blood of guinea pigs inoculated with the blood of infected soldiers. [2] But it was published later about 8 months after publication by Inada's group.

The name *'Leptospira'* for these organisms was coined by Hideyo Noguchi in 1918 following detailed microscopic and cultural observations. [3]

Figure 2:

Hideyo Noguchi

(Courtesy: Museum, Department of Microbiology, Sri Devaraj Urs Medical College)

In the next fifteen years after naming of *Leptospira*, many of the important serovars prevalent throughout the world and their host sources were discovered. [2, 13]

The association of rats in the transmission of leptospirosis was proved in the early part of 20th century. [16, 17] Rats have been thought to be associated with transmission of many diseases since ancient times. Falling of dead rats and association with plague has been described in biblical literature in the era before Christ. Also, Bhagavath purana describes it. The legend of *Pied piper of Hamelin* who piped all the rats from the town into the river Weser is placed at or about 1284. [18] The English poet, Robert Browning who lived during 19th century describes rats in his poem titled "*The pied piper of Hamelin*" as, [19]

Rats!
They fought the dogs and killed the cats
And bit the babies in the cradles,
And ate the cheeses out of the vats,
And licked the soup from the cook's own ladles,
Split open the kegs of salted sprats,
Made nests inside men's Sunday hats,
And even spoiled the women's chats
In fifty different sharps and flats.
Into the street the piper stept,
To blow the pipe, like a musical adept,
And out of the houses the rats came tumbling
And step for step they followed dancing,
Until they came to the river Weser,
Where in all plunged and perished!

Figure 3: Pied Piper of Hamelin

This poem also says how the rats were a menace. However, the perishing of the rats may not be the end of the story, as the water of the river Weser contaminated by urine of dying rats would always carry the risk of Leptospirosis.

Inada and his colleagues during the research on isolation of *Leptospira*, also studied about the pathogenesis and excretion of these organisms in the urine of patients and experimental animals. They demonstrated the organisms in the urine and faeces of infected rats, rabbits and guinea pigs by dark ground microscopy. Ido and his group in 1917 proved the role of rats in the transmission of the disease to humans that led to the establishment of leptospirosis as a zoonotic disease. [16] Also, the association of rats with Leptospirosis was worked on extensively during 1920, in few studies from different parts of the world and the association was proved. [17]

The first report of bovine leptospirosis was published in the former USSR in 1935 where *Leptospira interrogans, serovar grippotyphosa* was isolated from calves with acute infection.[13] The first isolation of *L. hardjo* from cattle was made by Roth and Galton in America, later in Canada by Robertson. In Australia, the first isolation of *L. hardjo* was made by Sullivan and Stallman. [2] Since then, further serovars were discovered throughout the world and now all pathogenic *Leptospira* are classified into one species *Leptospira interrogans* containing 258 serovars arranged into 25 serogroups. [20] The contribution of Faine and his group in the understanding of the basic biology of these organisms, identification of various serovars and the range of mammalian hosts, including humans, wild and domestic animals contributed significantly to the field of leptospirosis. [3, 21]

In the next two decades after this, the researchers tried to cultivate *Leptospira* in the artificial culture media containing 10% rabbit serum. But as the yield was very less and slow, researchers had significant impediments. It was difficult to understand the nutritional requirement of this organism in comparison with other bacteria.[1, 3] It was only in 1960 when J B Wilson developed a new culture media, the *Leptospira* were able to be grown with good yields. J.B Wilson was a laboratory physician in

Wisconsin, USA. During his research on *L.pomona*, which caused widespread leptospirosis in cattle and pigs in USA, he developed the Bovine albumin oleic acid culture medium which improved the yields. [3] Later, research continued and serological classification of Leptospires was done by means of agglutination reactions with specific antiserum. This classification was used for a long time till 1994, when genetic classification system was postulated. Currently, the genetic classification is in use. [22] ELISA method for leptospirosis was developed in 1980. [23]

Also, the work and research on leptospirosis has been supported by veterinary public health sources and veterinarians across the world. [3]

Studies have shown that *Leptospira* vaccines can prevent the disease. Efficacy of vaccine for preventing leptospirosis was shown soon after *Leptospira* was proven to be a causative agent of Weil's disease in Japan. Initial preparation was heat killed whole cell vaccine; however, specificity for serovar limits the efficacy of these vaccines. Leptospiral antigens that induce cross-protective immunity to the various serovars are sought as new vaccine candidates. Recent advancements include recombinant outer membrane protein (OMP) vaccines, Lipopolysaccharide vaccines, inactivated vaccines, attenuated vaccines and DNA vaccines. [24, 25]

Leptospirosis in Andaman Islands:

Andaman Islands assume an important place in the history of Leptospirosis in India and its research. Reports of Weil's disease had started appearing in the literature by the end of 19th century. Chowdary reported a series of jaundice cases that occurred over a decade starting from 1892 in Andaman which is believed to be Leptospirosis. However, the organism could not be demonstrated in these patients. In 1931, the first report of bacteriologically confirmed cases of leptospirosis originated from the

Andaman Islands. An extensive and detailed account of the disease was documented by Taylor & Goyle in the Andaman. Twenty isolates of Leptospires were obtained within a period of four months from 64 patients in Port Blair and surrounding villages. Reports of leptospirosis from many parts of India like Calcutta, Assam and Mumbai followed the discovery of leptospirosis in Andaman. [26] From 1980 till date, there were many reports of this disease from various parts of India. In south India, the states of Tamil nadu and kerala have reported several outbreaks. [27, 28, 29] Presently, leptospirosis has emerged as an important public health problem in India.

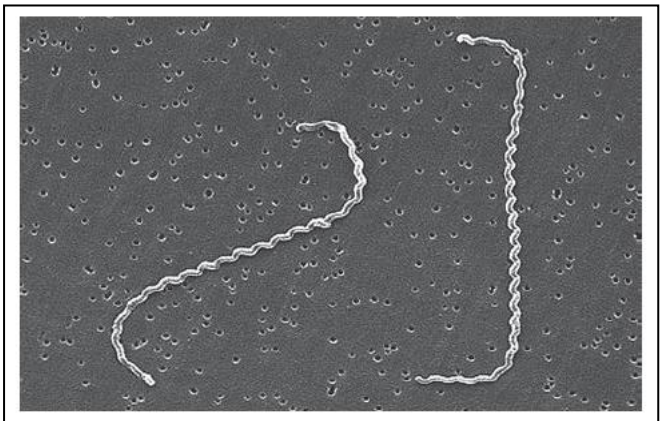
TABLE 1: HISTORICAL MILESTONES IN LEPTOSPIROSIS

Year	Scientist/place	Discovery
1886	Adolf Weil	Described icteric leptospirosis
1907	Stimson	Observed the organism in postmortem liver tissue by silver stained sections
1914	_	Saprophytic spirochaetes were described
1915	Inada and Ido in Japan 2 German physicians	Isolated the causative agent of leptospirosis
1918	Noguchi	Proposed the name '*Leptospira*'
1935	Former USSR	First report of bovine leptospirosis
1960	Roth and Gatton in America	Isolated *L.hardjo* from cattle
1964	Robertson etal in Canada	Isolated *L.hardjo* from cattle
1966	Torten etal	Described Indirect Immunofluorescence test
1969	Sullivan and Stallman in Australia	Isolated *L.hardjo* from cattle
1972	Abdussalam etal	Developed agglutination reactions
1973	Cole etal	Described Microscopic agglutination test
1980	Adler etal	Developed ELISA
1994	Abdussalam and Ellis etal	Current system of genetic classification

3. B: MORPHOLOGY

Leptospira are named so, as they are thin and spiral organisms. 'Leptos' means fine and 'spira' means coil in Greek language. The coils are tightly wound unlike that of the spirochaetes in the Genera *Treponema and Borrelia*. They are Gram negative bacteria measuring 0.1µm in diameter and 6-20 µm in length. [1, 3] As they are so thin, they can pass through routinely used membrane bacterial filters which are of size 0.2 µm. Also, they cannot be visualized by conventional light microscopy as only structures which are of size greater than 0.3 µm are seen by light microscopy. Thus, dark field microscopy offers an advantage to visualize *Leptospira*. They have characteristic hooked ends with either one or both ends bent in the form of a hook resembling question mark. [1, 2, 3] The morphology is shown in Figure 4.

Figure 4: Morphology of leptospira



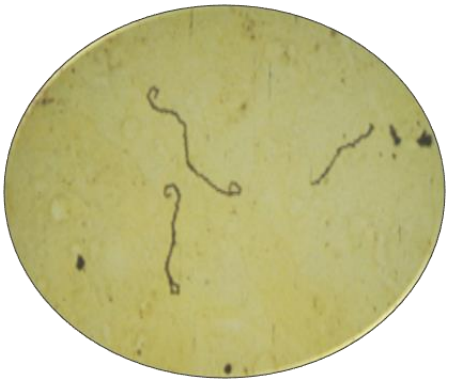
(Courtesy: Mandell Douglas, Bennett's Principles and practice of Infectious Diseases)

Staining methods: *Leptospira* are usually well stained with Geimsa's stain in which they appear red/ purplish red. [2] Flagellar stains like Leifson's stain and Gray stain, silver impregnation staining methods like Fontana's staining for cultures and

levaditi's staining for tissue sections, make them appear thicker, thus making light microscopic examination of the organism possible. This is shown in Figure 5.

Figure 5: Fontana's staining of *Leptospira*

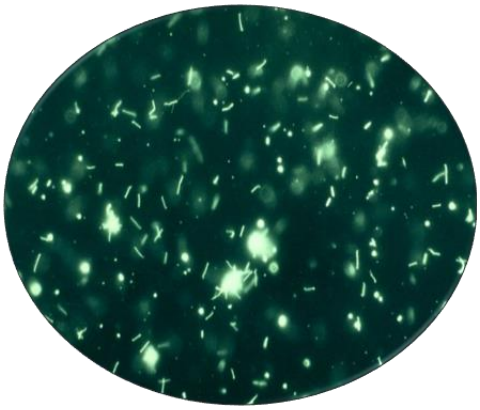
(Courtesy: Practice. bmj. Com)



Routine method of observation of *Leptospira* is by dark field microscopy of wet preparations. Leptospires appear as bright, flexible organisms. Sometimes, they appear as a series of small dots. [1, 3] They are actively motile and show characteristic cork screw and spinning movement; they spin fast along their long axis, and also show spiral/snake like movements. [1, 3] This is shown in Figure 6.

Figure 6: Dark field microscopic appearance of *Leptospira*

(Courtesy: Mandell Douglas, Bennett's Principles and practice of Infectious Diseases)



Phase contrast microscopy can also be employed for visualization of Leptospires in wet preparations. They are seen as thin, grey, spiral structures which are actively motile and sometimes as black dots. [1]

Electron microscopy reveals structural details. *Leptospira* resemble other bacteria in their cellular organization. They have cell wall, cytoplasmic membrane, cytoplasm with nucleus. They appear as protoplasmic cylinders covered by a cell membrane, cell wall and enveloped by a sheath. [3] Surface architecture resembles gram negative bacteria. However, cell wall shares the properties of both gram negative and gram positive bacteria. [21, 30] The cell wall consists of outer membrane, periplasmic region and the inner cytoplasmic membrane. Outer membrane consists of peptidoglycan layer, lipopolysaccharides and outer membrane proteins. Peptidoglycan layer is closely associated with the cytoplasmic membrane, unlike in other gram negative bacteria where it is located close to the outer membrane. [21, 31] Lipopolysaccharides (LPS) are highly antigenic and variations in these antigenic structures give rise to large diversity of the serovars and serotypes seen among the *Leptospira* species. [3]

Leptospira have got two internal flagellae or endoflagellae in contrast with three flagellae in *Treponema*. They arise from each end of the organism which traverse in the periplasmic region towards the centre. These endoflagellae are responsible for the cork screw and spinning movement of these organisms. [32]

Leptospira have different outer membrane proteins. Three of them have been studied well. They are transmembrane proteins, lipoproteins and peripheral membrane proteins. The porin OMPL1 is the first OMP described and is a transmembrane protein present as a trimer. The second class of OMPs, the lipoproteins constitute the most abundant of the leptospiral proteins in the outer membrane, to which they are anchored by fatty acids. Lipoproteins include Lip L_{32}, Lip L_{36}, Lip L_{41}, Lip L_{31}, Lip L_{21}, Lip L_{45} and Lip L_{48}. Among these, Lip L_{32} and Lip L_{41} are expressed by many pathogenic Leptospiral serovars during infection. The third class of proteins,

peripheral membrane proteins include the protein $P31_{Lip\ L\ 45}$ which can be released from membranes. [21, 33]

Leptospira, whether pathogenic or saprophytic contain 3 kinds of RNAs: 23S rRNA, 16Sr RNA and 5Sr RNA similar to the other prokaryotic organisms. [3, 34] However, in some *L. interrogans* strains, the classical 23S rRNA is further processed to generate 14S rRNA and 17S rRNAs. This unique processing step occurs in eukaryotes and a few prokaryotes. [35]

Non pathogenic Leptospires have three chromosomes whereas the pathogenic Leptospires have two chromosomes. The two chromosomes in pathogenic Leptospires include small replicons and large replicons. The large replicon has 4330 kb and the small replicon has 358 kb. The essential genes are found on small replicons and it is considered as the significant chromosome. [3, 36] It is thought that the higher gene density in *L.interrogans* may confer the ability of survival in this organism. [3]

It has been observed that in *L.interrogans serovar Lai*, 54 kb DNA fragment can isolate itself from bacterial chromosome and form a circular plasmid in the cytoplasm. This plasmid has been reported to constitute a genomic island containing a high proportion of novel genes. The presence of the plasmid is suggestive of the existence of mechanism for spreading of the genes from one organism to the other. [37]

The evidence for the horizontal transfer of DNA among *Leptospira* species comes from studies which showed changes in the intervening sequences found within 23SrRNA gene. Recombination events have been known to play a role in the organization of Leptospiral genome. These recombinant events are brought about by repetitive transposase- encoding insertion sequences which lead to genetic rearrangements. The mobility of these insertion sequence elements is responsible for the classification of different groups within the isolates. [3, 38]

3. C: CULTIVATION OF *LEPTOSPIRA*

Leptospires are obligate aerobes. As they are environmental organisms, they grow well at temperatures between 28 - 30°C. The growth is relatively slow with a generation time of approximately 6-8 hours. They take 3-4 weeks to grow in cultures and sometimes as long as 12-13 weeks. They have simple, but unique nutritional requirements. They utilize long chain fatty acids as the carbon and energy source and require vitamin B_2 (riboflavin) and vitamin B_{12} (cyanocobalamin) as growth factors. They also require ammonium salts. Tween 80 is commonly used in artificial culture media as the source of carbon. It is also necessary to include bovine serum albumin (BSA) which binds the free fatty acids that are toxic to these organisms. Rabbit serum at a concentration of 10% is added in certain media as a rich source of vitamin B_{12} and albumin which promotes the growth of these organisms. Leptospiral cultures are very prone for contamination with other bacteria and fungi. It is a frequently encountered problem in *Leptospira* laboratories. Antibiotics like 5-fluorouracil are incorporated into the culture media to avoid such contaminations. [1, 2, 3]

Several liquid media containing rabbit serum have been described by Stuart, Fletcher, korthoff and Noguchi. The most widely used conventional medium is Ellinghausen-McCullough-Johnson-Harris (EMJH) medium which contains bovine serum albumin and Tween 80. [39] These media can be rendered selective by addition of 5-fluorouracil or neomycin. [2,3] In liquid media, growth is seen as granular or as a deposit at the bottom. Liquid media are used for propagating the organisms. They are also used as a source of antigen in microscopic agglutination test as well as an antigen for coating the slides in indirect immune fluorescence test. [7] Leptospires can grow in protein free media. Such media become essential for vaccine preparation. [2, 3] The protein free media used for the purpose of vaccine production contains Tween 80

detoxified with charcoal which acts as a carbon source, vitamin B_1 (Thiamine) and vitamin B_{12} (Cyanocobalamin) along with inorganic salts and organic buffer. [40]

Long term maintenance is done in semisolid medium containing 0.2-0.5% agar. *Leptospira* take a long time to grow as stated earlier. In semisolid media, they grow just beneath the surface and appear as an opaque mass which has been given the name 'Dinger's ring or disc.[1] Solid media have been used for isolation of Leptospires, to separate mixed cultures of Leptospires, and for detection of haemolysin production. They can be stored for a long time at -70°c and also by lyophilization. [2]

Bact ALERT mycobacterial (MB) and enriched MB Bact ALERT blood culture systems have been shown to support the growth of Leptospires. These blood culture media are readily available in most of the clinical laboratories and thus they seem to provide a feasible method for inoculation of blood cultures on bedside in patients with suspected leptospirosis for further processing and identification. [41]

3.D: CLASSIFICATION OF *LEPTOSPIRA* AND GENUS DEFINITION:

Taxonomically, genus *Leptospira* are classified under the order spirochaetales and family leptospiraceae. Genus *Leptospira* includes organisms which are Gram negative, thin, spiral and difficult to visualize. They produce oxidase and are chemoorganotrophs. [3]

The different members of the genus *Leptospira* are classified by serological and molecular methods based on their antigen and DNA relatedness respectively.

Serologically, leptospires have been classified into two species. Pathogenic *Leptospira* are grouped under the species *L.interrogans* and non pathogenic *Leptospira* are grouped under the species *L.biflexa*. Pathogenic *L.interrogans* includes

about 268 serovars depending on the dominance of one or more epitopes in a surface mosaic of lipopolysaccharide antigens. Serovars that share common antigens and partially coagglutinate are grouped under one common sero group and 25 such sero groups are described. There appears to be an association of some serovars with certain animal species. [3, 20] The non pathogenic *L.biflexa* are saprophytes which are found in stagnant water, small water streams and lakes. They include about 60 serovars. [21]

The current classification of leptospira is based on genetic relatedness. They are classified into number of species by DNA reassociation studies and by 16 s rRNA sequencing. [22, 31] These include 7 saprophytic species, 8 pathogenic species and an additional 5 species with unclear pathogenecity. [21] This is shown in Table 2.

Table 2: Genetic classification of *Leptospira*

Saprophytic species	Pathogenic species	Species with unclear pathogenecity
L.biflexa	*L.interrogans*	*L. inadai*
L.wolbachii	*L.kirschneri*	*L. broomii*
L.kmetyi	*L. borgpetersenii*	*L. fainei*
L.meyeri	*L.santarosai*	*L. wolffii*
L.vanthielii	*L. noguchii*	*L.licerasiae*
L.terpstrae	*L.weilii*	
L. yanagawae	*L.alexanderi*	
	L. alstoni	

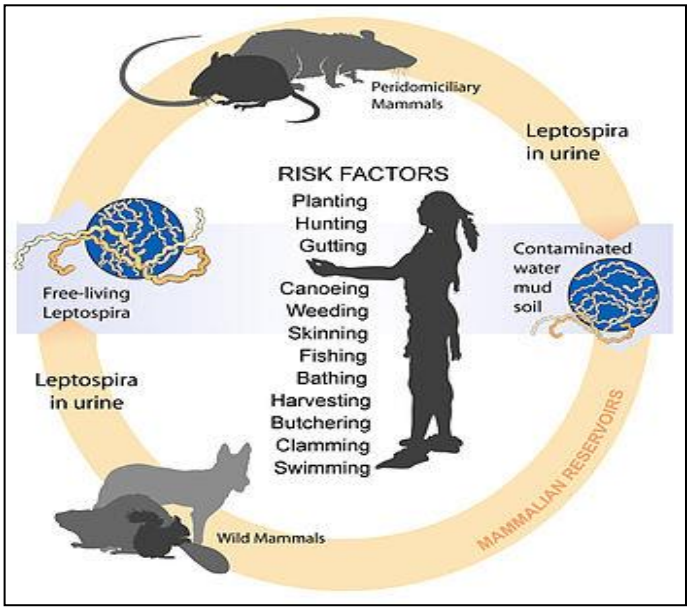
Molecular classification allows for the clear identification of distinct subtypes, however, the serological classification is widely used for epidemiological purposes. [2]

3.E: TRANSMISSION

Leptospira are transmitted from animals to humans. Man to man transmission does not occur. The mode of transmission to humans can be direct or indirect. Direct transmission occurs when *Leptospira* from urine, body fluids and tissues of acutely infected/ asymptomatic carrier animals enter the body of human beings and initiate infection. This occurs when man comes in direct contact with the animal. Indirect transmission occurs when an animal or human being comes in contact with environmental sources like soil and water contaminated by urine of excretor animals. This is considered the most common mode of transmission in general population. Rarely, infection may follow animal bites. [1, 2]

Infection occurs through various environmental vehicles. Three important factors determine the transmission. Firstly, the animal carriers, second factor is the suitable environment for the survival of *Leptospira* and the third factor is the exposure of human beings to the contaminated urine. [2]

Figure 7: Transmission of leptospirosis



(Courtesy: bezhare. Blogspot. Com)

Leptospires cannot be transmitted by ingestion, as the organisms cannot survive in acidic pH of the stomach. The organisms enter through minor abrasions or cuts in the skin or via the conjunctiva and intact mucosa. Man to man transmission is very rare. Leptospiral infection in man is a dead end of transmission. However, transmission by sexual intercourse during convalescence has been reported. Direct transmission among animals can be transplacental, through sexual contact or by sucking milk from infected mothers. Presence of *Leptospira* in the genital tracts as well as the transplacental transmission has been demonstrated in animals. [2, 20] Though, contact plays an important role in transmission, transmission by other rare routes like inhalation, ingestion, inoculation by animal bites and sexual contact also exist. [2]

3.F: PATHOGENESIS

After entering the body, Leptospires disseminate through blood to various organs and penetrate various tissue barriers. The incubation period of leptospirosis ranges from 2-20 days, but usually it is 7-10 days.

Once Leptospires are present in the blood vessels, they cause inflammation of the vessels leading to vasculitis. They also attach to vascular endothelial cells and bring about endothelial damage. Outer envelope of Leptospires possesses an antiphagocytic component, thereby escaping phagocytosis by macrophages. They are phagocytosed only in the presence of specific antibody. Inhibition of macrophage activity increases sensitivity to infection. [42] Virulent Leptospires become associated with neutrophils, but are not killed. Leptospiral LPS stimulates adherence of neutrophils and platelets to endothelial cells causing aggregation and suggesting a role in the development of thrombocytopenia. [1, 2]

There are 3 important virulence mechanisms, [43]

1. Immune mechanisms
2. Toxin production
3. Adhesins and other surface proteins

Immune mechanisms: Human toll like receptor (TLR4), which responds to extremely low concentrations of Gram negative lipopolysaccharide (LPS), is unable to bind Leptospiral LPS because of the unique methylated phosphate residue of its Lipid A. Immune mediated mechanisms influence the severity of symptoms; Human leucocyte antigen (HLA) DQ6 is an independent risk factor for leptospirosis. Leptospires produce a superantigen that can cause non specific T-cell activation in susceptible individuals. Other immune mechanisms that play a role in pathogenesis include, circulating immune complexes, anticardiolipin antibodies, antineutrophilic cytoplasmic antibodies and antiplatelet antibodies. Cytokines like TNF – α liberated in response to leptospiral LPS is also described to play a role. [2, 30]

Leptospira elaborate different types of toxins like haemolytic toxins, sphingomyelinases and phospholipases which cause tissue damage directly. They also have endotoxic activity due to LPS component. [2]

Adhesins and other surface proteins: Major surface lipoprotein is Lip L_{32}, which is a major target of human immune response. It mainly brings about tubulointerstitial nephritis. Another surface protein, lig protein mediate interactions with fibronectin, fibrinogen and other extracellular matrix proteins. They are also the important early antigens for the production of IgM antibodies. Endostatin like Len A (Leptospiral endostatin A) protein binds the complement regulatory protein, Factor H, suggesting an important role in the escape of complement mediated lysis. Endostatin

is a mammalian protein which is a proteolytic fragment of collagen and is a potent anti-angiogenic factor. [31]

Leptospires after reaching various organs of the body through blood, bring about different pathological changes in these organs by various pathogenic mechanisms as described above. Leptospires have been shown to attach to epithelial cells. Virulent Leptospires adhere to renal epithelial cells in vitro, and adhesion is enhanced by sub agglutinating concentrations of homologous antibody. After the organism gains access to the kidney, it migrates to the interstitium, renal tubules and tubular lumen causing interstitial nephritis and tubular necrosis. When renal failure develops, it is usually due to tubular damage, but hypovolaemia from dehydration and from altered capillary permeability can also contribute to renal failure. [44] Liver involvement is seen as centrilobular necrosis with proliferation of kupffer cells. Jaundice may occur as a result of hepatocellular dysfunction. *Leptospira* may invade skeletal muscle causing oedema, vacuolization of myofibrils and focal necrosis. Muscular microcirculation is impaired and capillary permeability is increased with resultant fluid leakage and hypovolaemia. In severe disease, a disseminated vasculitic syndrome may result from damage to the capillary endothelium accounting for broad spectrum of clinical illness. Vasculitis leads to pulmonary haemorrhage, ischaemia of the renal cortex and tubular epithelial cell necrosis and destruction of the hepatic architecture, and liver cell injury with or without necrosis. [2]

They may also invade the aqueous humour of the eye, where they may persist for many months leading to chronic or recurrent uveitis; immune complexes are thought to cause inflammation in ocular tissues. In horses, antibodies produced against epitopes of equine strains are known to cause recurrent uveitis. Similar

autoimmune mechanism may exist in humans. [45, 46] Also in CNS leptospirosis, immune mechanisms have been postulated to bring about pathogenesis. [2]

Despite the possibility of severe complications, the disease is most often self limited and non fatal. Over the period of time, a systemic immune response may eliminate the organism from the body but may also lead to a symptomatic inflammatory reaction that can produce secondary end organ injury. [2, 42]

3.G IMMUNOLOGY [2, 3, 26]

Immune response in leptospirosis is largely humoral in nature. Passive immunity can be conferred by antibodies alone. Immunity is strongly restricted to the homologous serovar or closely related serovars. Serovar specificity is conferred by the LPS antigens. Broadly reactive genus specific antigens have also been described. Outer membrane proteins are the potential for subunit vaccines which can generate broadly cross protective immunity. Few studies have reported cell mediated immune responses.

Antibodies usually develop within 2-12 days after the onset of illness. IgM antibody is the first to appear as in any other acute infections. They appear in the blood as early as on 4^{th} or 5^{th} day of the illness. They reach peak levels during 3^{rd} or 4^{th} week and start declining after 4^{th} week. They become undetectable within 6 months. IgG antibodies appear at about 4^{th} week after the onset of illness and are present at low levels for many years.

Microscopic agglutinating antibodies are different class of antibodies produced against the infecting serovar. They often react only with a certain serovar or serogroup. Agglutinating antibodies can be of both IgM and IgG classes. They appear

in detectable levels at the end of the first week of illness and reach peak levels during 3rd or 4th week and then decline slowly over months. They may present at low levels for a long time. 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 MAT.

3.H PATHOLOGY

Leptospirosis is characterized by the development of vasculitis, endothelial damage and inflammatory infiltrates. Inflammatory cells include monocytes, plasma cells, histiocytes and neutrophils. [2]

On gross examination, petechial haemorrhages are common in various organs and may be extensive. Also, the organs are often discoloured due to icterus. [2]

Histopathological changes are most marked in the liver, kidneys, lungs and heart. Rarely, other organs are affected. Liver shows intrahepatic cholestasis, hypertrophy and hyperplasia of kupffer cells and erythrophagocytosis. Changes in the kidneys include interstitial nephritis and intense intracellular infiltration by neutrophils and monocytes. Tubular cell brush borders are denuded and tubular basement membrane is thickened. Glomerular changes are also seen. Characteristic feature is that the Leptospires are seen within the renal tubules. Lungs show pulmonary congestion and haemorrhages. Alveolar spaces are infiltrated by monocytes and neutrophils and there is formation of hyaline membrane. Sometimes, the organisms are seen attached to capillary endothelial cells in the interalveolar septae. If the heart is involved, there is interstitial myocarditis characterized by plasma cells and lymphocytic infiltration. It may also show features of coronary arteritis. Skeletal muscle changes include focal necrosis of isolated muscle fibres and

inflammatory reaction with histiocytes, neutrophils and plasma cells. Involvement of brain shows perivascular cuffing. [1, 2, 3]

In anicteric leptospirosis, ESR is elevated, and WBC counts range from below normal to moderately elevated. Liver function tests show a slight elevation in aminotransferases, bilirubin, and alkaline phosphatase in the absence of jaundice. Urinalysis shows proteinuria, pyuria, and often microscopic haematuria. Hyaline and granular casts may also be present during the first week of illness. Lumbar puncture will usually reveal a normal or slightly elevated CSF pressure and may serve to reduce the intensity of headache. CSF examination shows lymphocytic predominance, protein may be normal or elevated, and glucose is usually normal. CSF abnormalities are common in the second week of illness, pleocytosis can persist for weeks. [2]

In severe or icteric leptospirosis, leucocytosis is seen. Thrombocytopenia is common and may be marked. [47] Renal function impairment is indicated by raised plasma creatinine levels. The degree of azotemia varies with severity of illness. [2, 44] In icteric leptospirosis, the increase in bilirubin is generally out of proportion to the other liver enzymes. Similar findings were reported for serum creatinine phophokinase levels. Serum amylase may also be elevated, particularly in patients with ARF. [2]

3.I CLINICAL FEATURES

Leptospirosis is a disease of protean manifestations. Clinical presentation ranges from mild febrile illness to fatal disease. Majority of infections caused by *Leptospira* are subclinical infections or of mild nature. They present with sudden onset of fever and other constitutional symptoms. The differential diagnosis include common viral infections such as, influenza, dengue, in addition to the bacterial

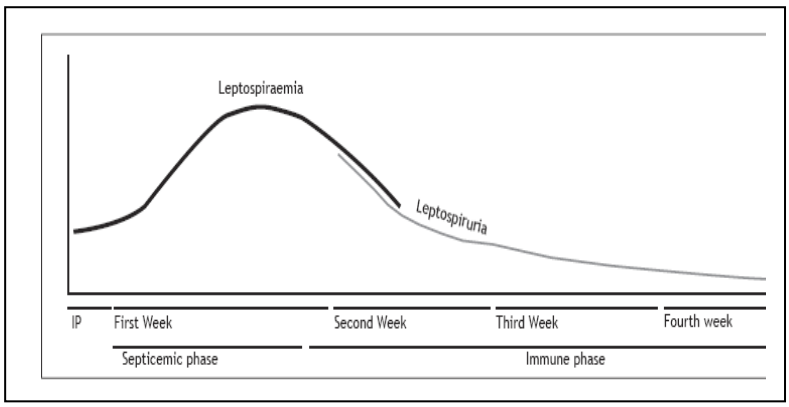
causes of fever of unknown origin such as typhoid, rickettsiosis, infectious mononucleosis, brucellosis, malaria, viral hepatitis and pneumonitis. Hanta virus infection must also be considered in the differential diagnosis in patients with pulmonary involvement. [2, 4]

The classical syndrome of weil's disease represents only the most severe presentation. It is characterized by fever, jaundice, hepatomegaly, splenomegaly and renal involvement. Serum bilirubin may become as high as 8-9mg/dl. Severe leptospirosis is most often caused by serotype *icterohaemorrhagiae*. It may also be due to *coppenhegani, bataviae, grippotyphosa, pyrogenes and lai*. [2, 48]

Distinct clinical syndromes are thought to be associated with specific serogroups; *canicola* with aseptic meningitis, *gryppotyphosa* associated with abdominal symptoms, *icterohaemorrhagiae* with jaundice. However, as the spectrum of illness produced by any serovar is quite wide, one cannot associate a particular serovar consistently with a disease involving a particular organ. [49]

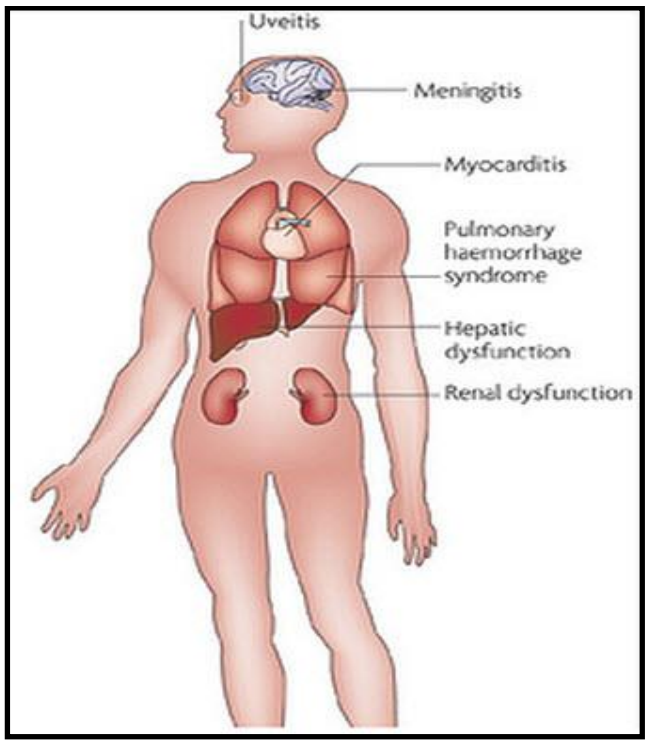
Clinically, Leptospirosis is classified into two types; icteric and anicteric leptospirosis. About ninety percent of the patients present with mild anicteric febrile illness. Ten percent are severely ill with jaundice and other manifestations. [2, 30, 48] Both anicteric and icteric leptospirosis may follow a biphasic course; an acute leptospiraemic or septicaemic phase that lasts for about a week, which is followed by an immune phase characterized by antibodies in the blood, disappearance of leptospires from blood and excretion of leptospira in urine.

Figure 8: Leptospiraemic and Immune phase of leptospirosis



Most of the complications of leptospirosis are associated with localization of leptospires within the tissues during the immune phase and thus occur during the second week of the illness. [2, 48] the clinical features of severe Leptospirosis is shown in the Figure 9.

Figure 9: Clinical features of severe leptospirosis



(Courtesy: www.microbiology bytes.com)

Anicteric leptospirosis is the most common and milder form of the disease. In the septicaemic phase, patients usually present with an abrupt onset of fever, chills, headache, myalgia, conjunctival suffusion, skin rash, nausea, vomiting and prostration. *Leptospira* can be isolated from blood, CSF and tissues. The fever is of high grade often reaching up to 40º C. Conjunctival suffusion usually appears on 3rd or 4th day. Myalgia usually involves the calf muscles, abdominal muscles and muscles of the lower back and can be severe. Skin rash is rarely seen in leptospirosis and if present is a transient urticarial, macular or maculo papular erythematous rash lasting for about 24 hours. The clinical presentation during this phase is nonspecific resembling other acute viral and bacterial infections as stated earlier. In the later part of anicteric phase, aseptic meningitis can occur especially in children. This septicaemic phase lasts for 3-9 days followed by a 2-3 days of defervescence, after which the immune phase develops. [48]

Immune phase is characterized by excretion of *Leptospira* in urine and correlates with the appearance of IgM antibodies in the serum. *Leptospira* settle in renal glomeruli and are excreted in urine. Fever and earlier constitutional symptoms recur in some patients. Signs of meningitis such as headache, photophobia and nuchal rigidity can develop which is more often seen in children. CNS involvement in leptospirosis most commonly occurs as aseptic meningitis. [2] A rare but severe manifestation is haemorrhagic pneumonia. [50]

Icteric leptospirosis occurs in 5-10% patients infected with *Leptospira* In this type of leptospirosis, the organisms disseminate from blood in to various organs resulting in multiorgan involvement which has a high mortality rate. The clinical presentation depends upon the predominant organ involved. Usually, there is jaundice, hepatomegaly, splenomegaly and renal involvement. The basic pathology is

vasculitis with endothelial damage and infiltration with monocytes, macrophages, polymorphs and plasma cells. [1, 2, 3]

Renal involvement leads to pyuria, haematuria, albuminuria, and granular casts. The pathology is interstitial nephritis with intense polymorphonuclear and monocyte infiltration. [2, 3]

Pulmonary involvement manifests with cough, breathlessness and haemoptysis due to pulmonary congestion and haemorrhage. Pathologically, infiltration of neutrophils and monocytes in to the alveolar spaces is seen. It has high mortality rate of up to 10-15%. Haemorrhagic pneumonia is a major presentation of leptospirosis most commonly seen in Andaman Islands. [2, 50]

Involvement of heart in leptospirosis is rare. It can cause myocarditis, coronary arteritis and pericardial effusion. ECG shows abnormal T waves. It has a very high mortality rate of 50%. [2]

Complications such as optic neuritis, uveitis, iridocyclitis, chorioretinitis and peripheral neuropathy occur most frequently in the immune phase. [2, 45]

Faine has evolved a set of criteria for diagnosis of leptospirosis on the basis of clinical, epidemiological and laboratory data. [1, 7] Certain necessary modifications have been made by Shivakumar etal in the epidemiological (Part B) and the laboratory criteria (Part C) of original Faine's criteria to make the diagnosis more practical in Indian institutions. In the Modified Faine's Criteria rapid tests (ELISA / SAT) have been introduced in Part C and rainfall has been included in Part B to make the diagnosis early and simple. [4, 51, 52] This is represented in the Table 3 below.

Table 3: Faine's and Modified Faine's criteria for Leptospirosis

Faine's Criteria		Modified Faine's Criteria	
Part A : Clinical Data		**Part A : Clinical Data**	
Question	**Score**	**Question**	**Score**
Headache	**2**	**Headache**	**2**
Fever	**2**	**Fever**	**2**
Temp > 39˚C	**2**	**Temp > 39˚C**	**2**
Conjunctival suffusion	**4**	**Conjunctival suffusion**	**4**
Meningism	**4**	**Meningism**	**4**
Muscle pain	**4**	**Muscle pain**	**4**
Conjunctival suffusion Meningism Muscle pain	**10**	**Conjunctival suffusion Meningism Muscle pain**	**10**
Jaundice	**1**	**Jaundice**	**1**
Albuminuria/Nitroge n Retention	**2**	**Albuminuria/Nitrogen Retention**	**2**
Total score		**Total score**	
Part B: Epidemiological factors		**Part B: Epidemiological Factors**	
Contact with animals or Contact with known contaminated water	**10**	**Rainfall**	5
		Contact with contaminated Environment	4
		Animal contact	**10**
		Total	
Part C: Bacteriological and Lab Findings		**Part C: Bacteriological and Lab Findings**	
Isolation of leptospira in culture – Diagnosis certain		**Isolation of leptospira in culture – Diagnosis certain**	
Positive Serology (MAT) Leptospirosis Endemic		**Positive Serology**	
Single positive – Low titre	**2**	**ELISA IgM Positive**	**15**
Single positive – High titre	**10**	SAT – Positive	**15**
		MAT – Single High titre	**15**
Leptospirosis Non Endemic			
Single positive – Low titre			
Single positive – High titre	**5**		
Rising titre (Paired sera)	**15**		
	25	**Rising titre (Paired sera)**	**25**

Presumptive diagnosis of leptospirosis is made by:

Part A or part A & part B score: 26 or more

Part A, B, C (Total): 25 or more

A score between 20 and 25 suggests leptospirosis as a possible diagnosis.

3.J EPIDEMIOLOGY

Leptospirosis is considered as the most widespread zoonosis in the world. It is primarily an infection of animals. Humans get infected accidentally. The natural habitat of Leptospires is the renal tubules of the carrier animals with chronic renal infection. Leptospires are maintained in nature by excretion in to the environment through the urine of these animals. [1, 2, 43] Man gets infected when he comes in contact with the urine of infected animals either directly or indirectly through contaminated soil and surface water. Although Leptospires are susceptible to various environmental factors like drying, acidic pH, salinity, presence of detergents and other bactericidal agents, they can survive for long periods in water and wet soil under favourable conditions. The organisms enter the host through the minor abrasions in the skin or intact mucous membranes of eye, throat and respiratory tract. [1, 2]

Once infected, carrier animals may excrete leptospira in their urine intermittently or continuously throughout their lives. Two types of hosts are recognized in *Leptospira*; reservoir hosts and carrier hosts. Reservoir hosts are those, in whom the carrier state lasts for lifetime. Rodents are the major reservoir hosts and these are the primary source of leptospirosis. They transmit infection both to the animals and human beings. Rodents are well known, worldwide reservoirs of serovar *icterohaemorrhagiae* associated with more serious, icteric type of leptospirosis. In the carrier hosts, the carrier state is temporary ranging from few months to years. Domestic animals such as cattle, dogs, and pigs are generally considered as carrier hosts. [2, 3]

Many studies have been done to show that rats are the major reservoirs. Study in Toronto, Canada during 1926 and 1927 demonstrated a prevalence of Leptospires in 37% of the rats that were studied. Also, a study from eastern Canada documented a

mean infection rate of 23% in Norwegian rats (Rattus norwegicus). Another study in British Columbia, during 1948 showed 4.8% prevalence in rats. A study from Detroit in 1983 reported that 90% of the rats were infected with *L.interrogans* serovar *Icterohaemorrhagiae.* [17] In India, four rodent species are found to be the major reservoir hosts of leptospirosis; *Rattus norwegicus* (Norway rat), *Rattus rattus* (House rat), Lesser bandicoot (*Bandicota bengalensis*) and Larger bandicoot (*Bandicota indica*). [30, 53] The association of certain animals with specific serovars is found to be significant; rats with the serovar *Icterohaemorrhagiae*, cattle with *Hardjo, Hebdomadis, Grippotyphosa*, dogs with *Canicola*, Pigs with *Pomona, Tarassovi* and *Bratislava.* [2]

The disease is seasonal with peak incidence during rainy season in tropical regions and during summer in temperate regions, where in, optimal temperature favours survival of Leptospires. Therefore, the illness commonly occurs during the monsoon months, during which, the infection is probably transmitted when humans come in contact with stagnant rain water contaminated by infected urine of animals. These organisms can survive for up to 6 hours in dry soil and for as long as 6 months in flooded condition. [2, 4]

Three epidemiological patterns of leptospirosis have been defined by Faine. [2] The first occurs in temperate climates where few serovars are involved and human infection occurs by direct contact with infected animals through farming of cattle and pigs. The second occurs in tropical wet areas, caused by many serovars infecting humans and animals and larger numbers of reservoir species, including rodents, farm animals, and dogs. Human exposure is not limited by occupation but results more often from the widespread environmental contamination, particularly during the rainy season. These are also the areas where large outbreaks of leptospirosis are most likely

to occur following floods, hurricanes, or other disasters. [54, 55, 56] The third pattern comprises rodent-borne infection in the urban environment. While this is of lesser significance throughout most of the world, it is potentially more important when the urban infrastructure is disrupted by war or by natural disasters. Outbreaks occurring in urban slum areas in developing countries have been reported. [57, 58]

Occupation is a significant risk factor for humans. Direct contact with infected animals accounts for most infections in veterinarians, abattoir workers, meat inspectors, rodent control workers, pet shop owners and other occupations which require contact with animals. Indirect contact causes infection in farmers [rice field workers, sugar cane cutters, fish farmers] sewer workers, septic tank cleaners, canal workers, miners and soldiers. Among the occupational risk groups, miners were the first to be recognized. The occurrence of Weil's disease in sewer workers was first reported in 1930s. Serovar *icterohaemorrhagiae* was isolated by inoculation of blood from patients and rats trapped in the sewers into guinea pigs and also from the slime lining the sewers. In Glasgow, Scotland, a seroprevalence among sewer workers of 17% was reported. [1, 2, 3]

Historically, leptospirosis has been given different names based on the occupational groups involved, clinical presentations and the place from where the disease was described. These are; seven-day fever in Japan caused by the serovar *L.hebdomadis*, cane cutter's or swine herd disease in Australia associated with *L.pomona*, rice field disease in Indonesia, mud fever/ marsh fever/ field fever in central Europe caused by *L.grippotyphosa* and yellow fever in temperate zones . [2, 30, 59] The association of fever with rats during the harvest has given the name 'harvest fever'. It has also been called mouse fever, fish handler's disease or water fever. [1]

Recently, it has emerged as recreational water borne disease in both temperate and tropical regions. Recreational activities like hiking, picknicking, swimming, canoeing and adventure race pose an increased risk in the transmission of Leptospires to man. Leptospires survive in natural, untreated water for months and years. [2, 17]

The British rowing champion Andy Holmes is reported to have died of Weil's disease on 24th october 2010. He was 51 yrs old. He became sick after competing in a 26-mile sculling marathon at Boston, Lincolnshire. He was admitted in ICU, and was put on ventilator support but succumbed to death. Andy Holmes rowed at the Olympics in 1984 at the Los Angeles and 1988 in Seoul Olympics and had won gold medals. [60, 61]

Figure 10: Andy Holmes – The Rowing champion

Leptospirosis is sporadic in temperate and desert regions where as, the disease is endemic in tropical areas and areas with high levels of subsurface water. Hence, China, Southeast Asia, India, Africa, South America and Central America are the regions which are endemic for leptospirosis. Large epidemics are reported after monsoons and heavy rainfall in this regions.[1, 2]

Based on the global data collected by International Leptospirosis Society (ILS) surveys, there are currently more >500,000 severe cases of leptospirosis annually. [62] Leptospirosis is now recognized as a globally re emerging disease with a marked increase in the number of cases in tropical countries than in temperate regions. [20, 63, 64] There are frequent outbreaks in Southeast Asia (Thailand, Malaysia, and Indonesia), India and the United States of America, particularly in Hawaii. [59, 62] Important outbreaks of leptospirosis and seroprevalence studies in India and the rest of the world are presented in the following Tables 4, 5, 6 and 7. [2, 59, 62]

Table 4: Important outbreaks of leptospirosis in the world

Year of study	Place	Study group	Risk factors
1961	United states	Army troops	Engaged in jungle exercise
1972	Uruguay	Cases of ARF	Recreational/ habitational exposure
1990	Hawaii	Cases with fever	
1995	Nicaragua	Epidemic haemorrhagic fever	Floods
1996	Laos	Acute jaundice cases	
1996	Costarica	Acute febrile disease	White-water rafters
1998	Wisconsin, Illinois	Acute febrile illness in Athletes	

Table 5: Important outbreaks of leptospirosis in India

Year of study	Place	Outbreak
1931	Andaman Islands	Leptospirosis among general population
1966	Andaman Islands	Acute febrile illness and jaundice in hospitalized patients
1985	Madras	Acute renal failure
1988	Madras	Fever, jaundice
1991	Madras	Acute febrile cases
1993	Diglipur, Andamans	Acute febrile disease with haemorrhagic manifestations
1994	Madurai	Uveitis
1999	Orissa	Acute febrile illness following floods
2000	Mumbai	General population following floods

Table 6: Seroprevalence of leptospirosis among different study groups in different parts of the world

Year of study	Place	Study group	Seroprevalence(%)
1975-1977	Barbados	Sanitation workers Sugarcane workers	42.7 39.4
1977	Barbados	Fever cases suspected of leptospirosis Healthy indivduals	28.7 15
1978	Trinidad	Sugarcane workers	45
1977-1982	Trinidad	Fever cases suspected of leptospirosis	9
1982	Somalia	General population	
1987	Italy	Rural areas Urban areas	11.34 3.08
1987	Karachi, pakisthan	Hospitalized patients with fever	25
1989	Barbados	School children	12.5
1995	Trinidad	School children	9.5
1995	Bolivia	Healthy individuals	30.5
1995	Turkey	General population Rice field workers	5.48 13
1996	Laos	Jaundice cases	21

Table 7: Seroprevalence of leptospirosis among different study groups in different parts of India

Year of study	Place	Study group	Seroprevalence(%)
1967	Bombay	Patients with febrile illness with suspected jaundiced Workers in animal farms and piggeries	30 50
1983	Madras	Cases of febrile illness Jaundice	18 24
1983	Madras	Children	47
1993	Madras	Conservancy workers	32.9
1995	Pondicherry	Febrile jaundice patients	12
1995	Calicut	Occupational groups Fishermen Sanitary workers Farmers Sewer workers	 52.8 56.2 30 28.2
1998	Bangalore	Patients with febrile illness in hospital	6
2007	Hyderabad	Cattle	36.4

In India, Kerala, Tamil Nadu and Andamans are known to be endemic for leptospirosis. But now with better facilities to detect the disease, the disease is being reported from almost all parts of India. [48, 59]

In a study conducted all over the country by National Reference Centre, Regional Medical Research Centre, Indian Council of Medical Research (ICMR), Port Blair during the period 2000–2001, a seropositivity rate ranging from 0 to 46.8 % amongst all cases of fever was observed from various parts of India. The positivity rate was highest in South India at 25.6%. It was 8.3%, 3.5%, 3.1% and 3.3% in northern, western, eastern and central India respectively. [48]

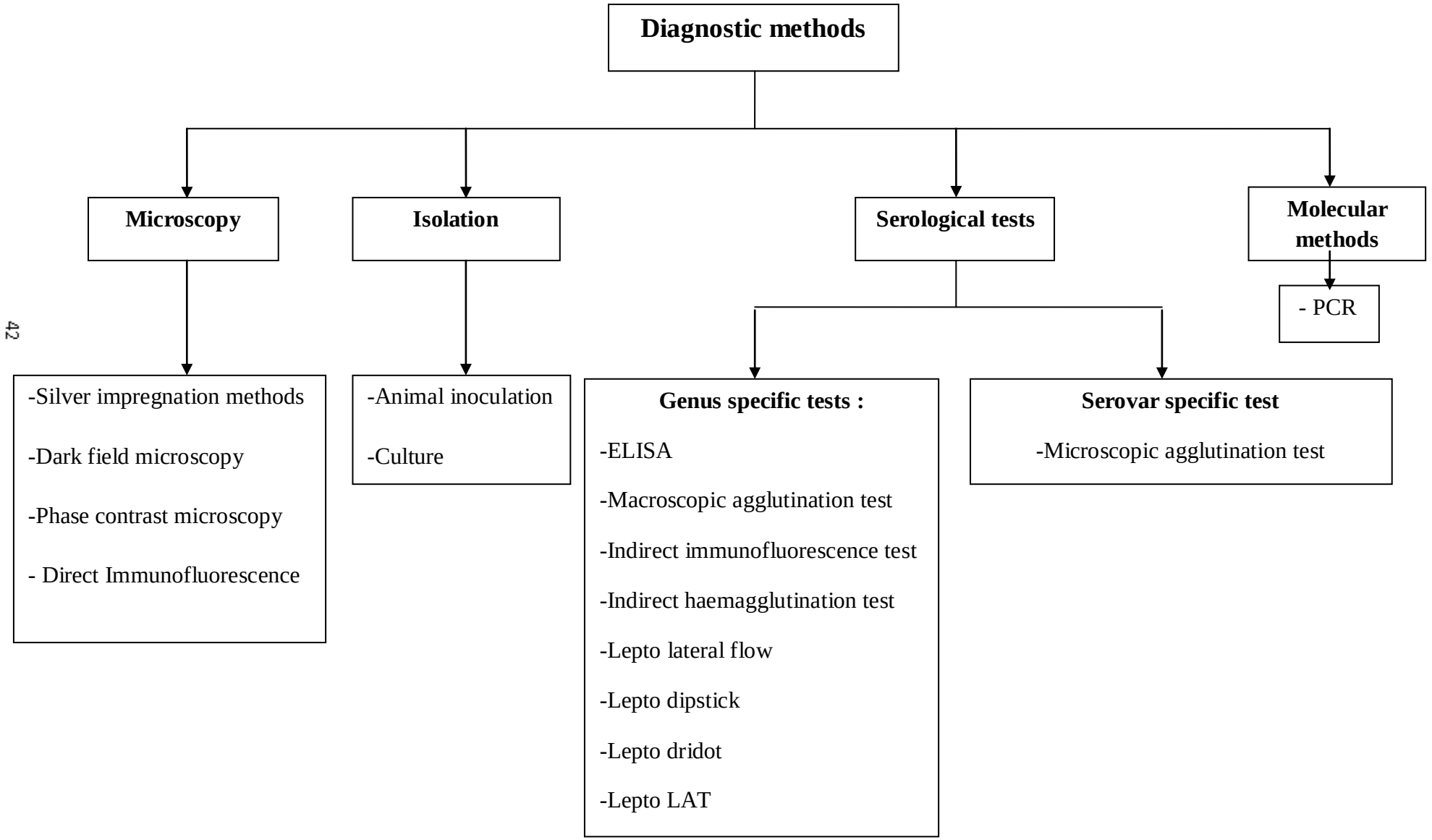
Leptospirosis in Karnataka:

Leptospirosis outbreaks have been reported from 15 districts of Karnataka. The highest incidence of cases has occurred in Bangalore city, Uttara kannada, Shimoga, Bidar, Gulbarga, Udupi and dakshina kannada districts. During the year 2004, 152 cases and 11 deaths were reported and during 2005, 224 cases and 19 deaths were reported. [4]

3.K LABORATORY DIAGNOSIS

As the clinical presentation of leptospirosis is varied and resembles many other common febrile illnesses, making clinical diagnosis is difficult. Therefore, accurate diagnosis requires laboratory support. Choosing the right specimens in right time and results of appropriate laboratory tests play an important role in making a proper diagnosis. It is also important to interpret the results properly. In this context, knowledge of different laboratory methods and their proper interpretation becomes important. Laboratory methods can be classified as follows as depicted in the following page in Figure 11

Figure 11: Laboratory Diagnostic methods for leptospirosis



Microscopy :

Though microscopy is easily performed and takes less time, it is most often considered to be non specific and insensitive. However, in the leptospiraemic phase of infection, where the antibodies are not formed in the body still, microscopy is useful. The diagnostic tests which detect antibodies cannot be used during this phase of infection.

Staining techniques: [2, 7, 26]

The staining techniques can be performed on patients' body fluids and tissue samples. These include,

1. Silver impregnation methods:

- Fontana's staining for fluid samples
- Warthin-starry stain for tissues

Well stained preparations show black spirochaetes in pale yellow or brown tissue elements.

2. Immunofluorescence staining

   This is used to demonstrate leptospires in bovine urine, water and soil, hence finding application in veterinary and environmental practice

3. Recent developments include

- Immunohistochemical method
- Immunoperoxidase staining

Dark field microscopy (DFM) [7, 26]

Leptospires cannot be observed under the ordinary light microscope and are so thin, that they cannot take up conventional stains. They can be visualized in the wet preparations under DFM. In DFM, a special condenser with a central circular stop is used. This allows only the oblique light rays to fall on the object, while the central light is interrupted. Due to the interruption of central light rays, only the oblique light rays which hit the object are diffracted and enter into the objective lens. The light rays hitting the suspending medium fail to diffract and hence do not enter the objective lens failing to reach the eyes. This results in standing out of leptospires as thin silvery threads against a dark background.

This procedure appears to be simple and rapid, but has several disadvanatges. Though the organisms are present in blood during the acute stage of the disease, the concentration of the organisms is too low, thereby making the detection difficult. *Leptospira* can be concentrated by differential centrifugation technique but the percentage of positive observations remains low. [7]

Serum protein, fibrin strands and other cell debris in blood resemble *Leptospira* due to their Brownian movement. Therefore great care and experience are necessary to avoid mistaking the other structures as *Leptospira*. [2] Urine sample has got the similar drawbacks in addition to the intermittent shedding of *Leptospira*. Also, the organisms are seen in urine only after 8-10 days of the onset of illness during immune phase. [2, 3]

Evaluation of this technique as a diagnostic tool has shown that the test has low sensitivity (40.2%) and low specificity (61.5%). Few studies have shown that the results obtained in DFM on samples from confirmed patients and controls were

identical. [7, 26] Therefore DFM is not recommended as a good diagnostic tool for the diagnosis of leptospirosis. In contrast, few studies opine that DFM can be considered as a good early diagnostic method in the settings where other diagnostic methods are unavailable. Also, as it is an easy and rapid technique, it can help in the early diagnosis and management of patients with suspected leptospirosis. Correlation and evaluation of DFM with MAT and ELISA for the diagnosis of leptospirosis in two studies from south India document that there was 95.8% efficacy when DFM was combined with IgM ELISA and 80% correlation between DFM and MAT. [65, 66]

Although it is not useful as a primary diagnostic method, it is helpful in visualizing growth of *Leptospira* in culture and monitoring the growth. Also, it is used to detect agglutination of *Leptospira* in microscopic agglutination test. [7, 26]

Phase contrast microscopy: [1]

Phase contrast microscopy is not very useful because its success depends on thin preparations and as the organisms' motility takes them rapidly in and out of the phase, it is difficult to visualize them. However, it is useful to observe granular forms.

Isolation:

Isolation of *Leptospira* from clinical specimens is the definitive diagnostic test for leptospirosis. But, the organism takes 4-6 weeks to grow and also sometimes, the growth is unreliable. Hence, this method is not useful in the management of patients. Prior administration of antibiotics in the patients greatly reduces the chances of isolation. [1, 2, 3]

The isolation and identification is the method of choice to identify circulating serovars in a particular geographical region. The samples from which, isolation can be

done include; blood and CSF during the first week of illness and, urine during the second week of illness for up to 1month. [26]

For isolation from blood, venous blood is collected by means of an aseptic technique and ideally inoculated at the bedside into blood culture bottles containing culture medium for *Leptospira*. Small inocula consisting of a few drops of blood are inoculated into several tubes, each containing 5 ml of a suitable medium. Large inocula will inhibit the growth of *Leptospira*. In case of urine, fresh midstream urine sample is collected and inoculated immediately; since urine is acidic and decreases the viability of *Leptospira*, it should be inoculated into the medium within 2 hours after voiding.

The most commonly used media for culture are EMJH medium with 5-fluorouracil, Fletcher's semisolid medium and Korthoff's liquid medium. The cultures are incubated at 28-30ºc in dark. *Leptospira* typically grow as a band 0.5-1 cm below the surface in semisolid medium and as granular deposit in liquid medium. The cultures are examined weekly by DFM for the presence of *Leptospira* up to 13 weeks before being discarded. Isolated *Leptospira* are identified by serological or molecular techniques. [7, 26]

Bact ALERT mycobacterial (MB) and enriched MB Act ALERT blood culture systems have been shown to support the growth of *Leptospira*. In a study, this was proved by inoculating *Leptospira* available from cultures. These media are readily available in most of the clinical laboratories and thus they seem to provide a feasible method for inoculation of blood cultures on bedside in patients with suspected leptospirosis. [41] However, presently, there are no studies employing clinical samples. Hence, further studies are necessary.

Culture has several disadvantages; *Leptospira* grow slowly, hence, their isolation 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 *Leptospira* is commonly encountered. The successful isolation rate is less due to prior use of antibiotics, imperfectly cleaned glass ware, using wrong incubation temperature and variations in pH. Fastidious organisms require special medium for isolation. Thus, culture is not employed as a diagnostic procedure in clinical practice. It is used for research purposes in reference laboratories, as a source of antigen for various serological tests and for vaccine production. [2, 3]

Serological tests:

Serological tests are the widely used methods for the diagnosis of leptospirosis. Various serological techniques are described and developed for the diagnosis of leptospirosis. Each one of these serological techniques has its own sensitivity and specificity. It is often necessary to use a number of techniques, either together or successively, to make a reliable and accurate diagnosis. [7] The enzyme-linked immunosorbent assay (ELISA) and the microscopic agglutination tests (MAT) are the serological tests generally used. Among these two tests, MAT is considered as the reference standard test. In the past few years, various rapid serological tests have been developed and the kits are available commercially. [7]

The interpretation of serological data always relies on the examination of serum samples sequentially; two serum samples collected at an interval of 8–10 days. These are called paired sera, one collected during the acute stage and the other during convalescent stage of the disease. Demonstration of sero-conversion or four-fold rise

or fall in titres among the paired samples is considered to be a confirmatory diagnostic test in serological practice. [7, 26]

Genus specific serological tests:

Enzyme linked immunosorbent assay (ELISA):

ELISA is one of the rapid serological techniques and is commonly used for the diagnosis of leptospirosis. Availability of commercial kits which are ready to use makes it a serological test of choice. The test can detect specific antibodies earlier than MAT, on 5thto 6th day of the disease, as it is more sensitive to IgM antibodies. Hence, it helps in the rapid diagnosis of current infection. [WHO] *L.biflexa serovar Patoc strain Patoc I* is used as the antigen. I_gM ELISA using this genus specific antigen is simple and more sensitive. 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. Several commercial kits to detect IgM and IgG antibodies are available from manufacturers. [7] The test may become negative more quickly than the MAT, although low levels of specific IgM may persist.

The ELISA is a very sensitive and specific test for the diagnosis of leptospirosis. It is of particular value as a serological screening test, because of its relative simplicity in comparison with the MAT. However, the test may be negative, e.g. in a large percentage of infections caused by serogroup *grippotyphosa* and, to a lesser extent, in the detection of serogroup *australis* infections. If a variety of strains from different serogroups as antigens are used instead of an antigen derived from the Patoc I saprophytic strain, the sensitivity of the test increases, but then, it becomes more cumbersome and difficult, as practically it is not possible to coat ELISA micro titre plates with so many different antigens. ELISA tests can also be used in

epidemiological studies to determine the seroincidence or seroprevalence of leptospirosis. [7]

ELISA is not highly specific. These tests are known to cross react with a range of non specific antibodies such as those in, Dengue, HIV infection, malaria, typhoid fever, Hanta virus infections and in some autoimmune diseases. [2, 8, 9]

To detect antibodies in the test serum, the serum is placed in contact with an antigen which is fixed on a solid support, namely a microtitre plate. Following an incubation period and numerous washes to eliminate excess antibody, an anti-species antibody (to which the test serum belongs) conjugated to an enzyme, is added. The enzyme activity is then determined by adding a specific chromogenic substrate. The intensity of the colour reaction, which is related to the quantity of degraded substrate, is proportional to the amount of antibody present in the test. The antigen antibody reaction is inferred visually or measured by spectrophotometer or ELISA reader. Finding out the ratio between sample optical density values and cut off value and multiplying it by 10 is expressed as ELISA units.

ELISA has several advantages over MAT. It detects antibodies earlier than MAT. Single antigenic preparation can be used. Antigens are heat stable and are therefore stable at room temperature for long periods. It is used for rapid processing of large number of samples. However, the test is less specific and the infecting serovar cannot be identified as the test will detect only the genus-specific antibodies. [7, 26]

Indirect Immunofluorescent Antibody (IIFA) technique:

A genus specific IIFA test was an early serological test described for the diagnosis of leptospirosis. It was sandardised by Torten et al, using a broadly reactive strain of *Leptospira*, the *patoc strain.* They reported it, as a rapid diagnostic test and

can be used for the early diagnosis of leptospirosis. It is useful in detecting recent infection and to guide the clinician in treating the patients. Before the advent of currently available wide array of serological tests, IIFA was considered as a good alternative method for the diagnosis of leptospirosis instead of the much laborious and tedious MAT. [67]

IIFA is a method using specific antigen-antibody reactivity to demonstrate antibodies in the sera and other body fluids in the presence of fluorescent dyes. It is a double antibody technique. The antibodies present in the test sample which bind to the antigens, are visualized by a fluorescent labelled anti antibodies directed at these antibodies. This is shown in Figure 12.

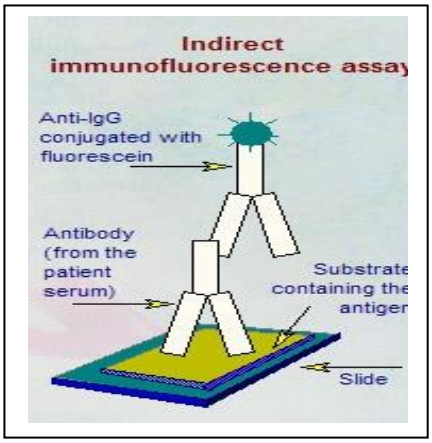


Figure 12:

Indirect immunofluorescence principle

Serial dilutions of test sera are mixed with *Patoc strain of Leptospira* which has been coated on a clean glass slide. The slide is incubated and washed as per the standard procedure. A fluorescein isothiocyanate conjugated rabbit anti-human immunoglobulin is added and examined under the fluorescent microscope for a visible fluorescence. A test is called positive only when obvious yellow-green fluorescence was emitted from morphologically intact *Leptospira*. End point titre is taken as the highest serum dilution giving a visible fluorescence. [67]

Torten and his colleagues compared the results of IIFA with MAT, which showed that IIFA is more sensitive. Similar opinion has been echoed by a study

conducted in India. [68] However, there are very few reports on evaluation of IIFA for rapid diagnosis of leptospirosis. Most of the studies have been done in Thailand and have shown that IIFA test appears to be a moderately sensitive and specific test. [10, 69, 70] In a prospective evaluation of 4 immunodiagnostic tests for human leptospirosis, IIFA showed highest sensitivity and specificity compared to other tests: MAT, latex agglutination test and Immunochromatographic test. [10] A very few studies have been done in other parts of the world including India. [68]

Other Genus specific serological tests: These include, a wide range of commercially available tests which are rapid, simple, and easy to perform and interpret the results. It does not require any special expertise or equipment. All these tests employ *Patoc strain of leptospira* as the antigen which is genus specific. These tests can be used as screening tests and need to be confirmed. The tests under this variety include:

Macroscopic slide agglutination test (MSAT) [7, 26]

This test is based on the principle of agglutination reaction and is similar to the other slide agglutination tests used for the diagnosis of other infectious diseases such as Rose Bengal agglutination test for brucellosis. 10 µl of test serum and one drop of antigen on a glass slide should be mixed thoroughly with an applicator stick and the slide should be rotated at 120 rpm for 4 minutes in a slide rotator.

The reaction is recorded as ++, when the clumps are large and definite with the suspension being clear, + 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 stable for six months at 4ºC to 8ºC. 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 is comparatively low. It may not be useful to detect the recent infection

Microcapsule agglutination test (MCAT) [7, 71]

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 adsorbed. These antigens react with the test serum containing antibodies to produce agglutination. The results are recorded as: positive - when the agglutination pattern cover the entire bottom of the tube, doubtful - when agglutination pattern is slightly larger than the negative control and negative - if no agglutination is observed.

Lepto dipstick assay [26, 72]

The assay is based on the binding of *Leptospira* specific IgM antibodies to the *Leptospira* antigen. Bound IgM antibodies are detected with an anti-human dye conjugate. The result is interpreted as positive when there is a clearly visible reddish coloured band which is again graded as 1+, 2+, 3+ and 4+ based on the intensity. It is considered as negative when there is no reddish coloured band.

Lepto lateral flow [7, 26]

Lepto lateral flow is an Immunochromatographic test. IgM antibodies bound to the broadly reactive antigen are detected with an anti human IgM gold conjugate contained within the test device [6-br-vij]. The results are interpreted as positive, if both

the test and control bands are stained. The test is negative, if only the control band is stained. Results are very quick and both serum as well as blood can be used to perform the test. It has the disadvantage that it is expensive.

Lepto dri-dot [26, 73]

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. 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.

Lepto-LAT [73]

Lepto–LAT is a latex agglutination test for the detection of antibodies to *Leptospira* in the body fluids. It is an agglutination immune assay for the detection of *Leptospira* specific antibodies. The blue coloured latex beads are sensitized with the broadly reactive and specific antigen prepared from the 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. The test is done by mixing 10 µl of latex reagent on a slide with 5 µl of test serum and rotating the slide slowly and gently. The results should be read within 60 seconds. The test is interpreted as positive when there is clearly visible granular agglutination and as negative when there is absence of agglutination.

Serovar specific test:

Microscopic agglutination test: (MAT)

MAT is the most commonly used serological technique for confirmation of diagnosis in leptospirosis. 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. [2, 7] However, this delays the diagnosis. The test is highly sensitive when performed on paired sera and is serovar or serogroup specific. But, the collection of convalescent serum sample is difficult in routine practice. Therefore, one of the critical issues of MAT is to find out or fix the cut-off or significant titre in the test for diagnosis, when only a single sample is available. The cut-off titre in MAT for a single sample depends on the baseline titre in the community in a particular geographical region. Different laboratories use different cut off titres for diagnosis based on endemicity or baseline titre in the community. Several investigators usually consider a titre of 1:80 as a significant titre for diagnosis without considering the endemicity or baseline titres in the community. This, sometimes may result in over diagnosis and overestimation of disease burden. [26, 75] Although MAT has several drawbacks, it is an accurate and reliable test in the diagnosis of leptospirosis as well as in seroepidemiological studies. The test is more specific and sensitive if performed on paired sera.

The MAT is based on the old agglutination-lysis test developed by Martin & Pettit and modified later by Cole and colleagues. [74] Both IgM and IgG class of antibodies take part in this test. It is difficult to standardize this procedure, as live antigens are often used and various factors, such as the age and density of the antigen culture, can influence the agglutination titre. Also, it is difficult to maintain many

different serovars in cultures in the routine laboratories. Hence, this test is carried out only in reference laboratories. [7]

The method consists of mixing the equal volumes of series of test serum dilutions and culture of *Leptospira* in the wells of microtitre plates and allowing serum antigen mixture to react for a period of 2 hrs at 37°C. The degree of agglutination and the endpoint titre is determined by examining a drop of the mixture under dark field microscopy. According to the Taxonomic Subcommittee on *Leptospira*, the end-point is defined as that dilution of serum which shows 50% agglutination, leaving 50% free Leptospires when compared with a control culture diluted 1:2 in phosphate-buffered saline. [7, 74]

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. [7, 26] Locally isolated strains should be included in the panel, if possible, as they may give more specific and sensitive results than the reference strains. At least one strain of saprophytic serovar (Patoc I) should also be included in the panel to act as a genus specific antigen to detect infections caused by serovars/strains that are 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 shown in the Table 8. [26]

Table 8: Recommended panel of serovars for MAT in India

SEROGROUP	SEROVAR	STRAIN
Andamana	Andamana	CH 11
Australis	Australis Bratislava	Ballico Jez Bratislava
Autumnalis	Autumnalis Rachmati	Akiyami Rachmat
Ballum	Ballum	S 102
Bataviae	Bataviae	V Tienen
Canicola	Canicola	Hond Utrecht IV
Celledoni	Celledoni	Celledoni
Cynopteri	Cynopteri	3522 C
Grippotyposa	Grippotyposa	Moskva V
Hebdomadis	Hebdomadis	Hebdomadis
Icterohaemorrhagiae	Icterohaemorrhagiae Copenhageni	RGA M 20
Javanica	Javanica Poi	Veldrat Bat. 46 Poi
Panama	Panama	CZ 214
Pomona	Pomona	Pomona
Pyrogenes	Pyrogenes	Salinem
Shermani	Shermani	LT 821
Sejroe	Sejroe hardjo	M 84 Hardjoprajitno
Semaranga	Patoc	Patoc 1
Tarassovi	Tarassovi	Perepelitsin

(Courtesy: Laboratory manual – Vijayachari)

MAT is a serovar or serogroup specific test. Hence information about the infecting serovar can be obtained. It has good sensitivity and specificity. Once infected, the person stays MAT Positive for several years, thus the test is useful for epidemiological purpose. [2, 7]

The test has several disadvantages as follows [26]

- It is technically exacting, about14–21 strains have to be maintained in culture which is often very difficult

- Test Procedure is complex and time consuming, reading results require 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 and a second serum sample is required for demonstrating seroconversion which is diagnostic. But obtaining second sample during convalescent phase is practically very difficult. It gives false negativity in the early course of the disease.

Molecular techniques:

One of the molecular techniques used for the early diagnosis of leptospirosis in recent years is the amplification of specific fragment of Leptospiral genomic DNA in clinical samples using polymerase chain reaction (PCR). Since the test detects specific fragment of Leptospiral DNA, the positive test result confirms the diagnosis. PCR can be applied to blood, urine, CSF and tissue samples. [7]

Nucleic acid probes and hybridization techniques were used previously but have been replaced by PCR. [2] Primers in combination with heat stable DNA polymerase in the presence of nucleotides subjected to temperature cycles, amplify a stretch of Leptospiral DNA. As a major target, the rrs gene encoding 16srRNA has been used. Other targets include the secY and flaB genes. [7] The amplified DNA can

be relatively easily detected in gels. Also, subsequent or concomitant hybridization with labelled probes makes the detection possible, during or after PCR.

The limitation of PCR based diagnosis of leptospirosis is the inability of most PCR assays in identifying the infecting serovars. While this is not significant for individual patient management, the identity of the serovar has significant epidemiological and public health value. [2] So, various molecular typing methods have been described. Methods employed have included the digestion of chromosomal DNA by restriction endonucleases (REA), restriction fragment length polymorphism (RFLP), ribotyping, pulsed field gel electrophoresis (PFGE) and a number of PCR based approaches. [2] PCR based DNA finger printing methods are being routinely used for characterizing Leptospiral isolates. These include; random amplified polymorphic DNA (RAPD) finger printing, arbitrarily primed PCR (AP-PCR), single nucleotide polymorphism of specific PCR product, repetitive extragenic PCR (REP PCR), fluorescent amplified fragment length polymorphism (FAFLP). [30, 73] FAFLP analyses of isolates from patients during epidemics and sporadic cases in Andaman Islands have shown that the outbreak associated isolates formed a single tight cluster.

3.L TREATMENT

Treatment of leptospirosis varies depending on the severity and duration of symptoms at the time of presentation. However, the basis of antibiotic treatment is the same. Preferably, the antibiotics should be started within 7-10 days of infection immediately on suspicion or diagnosis of leptospirosis, because the organ damage which occurs in the second week of illness will not be influenced by antibiotic treatment. [30, 48, 76]

Patients who present with mild flu-like symptoms in anicteric leptospirosis do not require admission to the hospital. They can be treated on outpatient basis, with oral Doxycycline 100mg twice a day for 7 days. Studies have shown that doxycycline is effective and it reduces the duration and severity of illness by an average of 2 days. [2] Patients who present with more severe anicteric leptospirosis like patients with features of aseptic meningitis, thrombocytopenia and others will require hospital admission and close observation. The antibiotic of choice is intravenous Benzyl penicillin in a dose of 5-6 million units/day for 5 days. [43] In patients who are allergic to penicillin, erythromycin 250mg, 4 times daily for 5 days is recommended. Alternatively, doxycycline 100mg twice daily for 10 days may be given. The management of patients with icteric leptospirosis, many a times require admission of the patients to the intensive care unit. Ceftriaxone or Cefotaxime are the antibiotics of choice. [43] Azithromycin 15mg/kg body weight twice daily has been tried and found to be effective in 72% of the patients. [77] Patients with pre renal azotemia require rehydration and their renal function should be monitored. Whereas, patients in acute renal failure require dialysis, either peritoneal or haemodialysis. Cardiac and respiratory monitoring is also required during the first few days of admission in ICU. Thrombocytopenia is usually self limiting. [2, 77]

3.M PREVENTION

The preventive measures to control leptospirosis include

a) Mass immunization of domestic livestock

b) Vaccination of humans who are at risk of developing leptospirosis

c) Rodent control and

d) Personal protective measures

Immunization: Immunity to leptospirosis is mainly humoral and is relatively serovar specific. Hence, immunization protects against disease caused by the homologous serovar or antigenically similar serovars only. Vaccines must therefore contain serovars representative of those present in the population to be immunized. [2]

Early vaccines consisted suspensions of of heat killed or formalin killed Leptospires grown in media containing animal serum, which regularly caused severe clinical reactions, mainly dermal toxicity and pyrogenicity. Modern vaccines prepared using Protein free medium are generally without such adverse effects. [24, 25, 40] In developed countries, pigs, cattle as well as domestic dogs are widely immunized. But in most developing countries, vaccines which contain the locally relevant serovars are not available. Most bovine and porcine vaccines contain serovars *hardjo and Pomona*, but few of them also contain serovars *canicola, grippotyphosa and icterohaemorrhagiae*. Canine vaccines generally contain serovars *canicola and icterohaemorrhagiae*. [2]

Human vaccines have not been widely used. Immunization with polyvalent vaccines has been practiced in Far eastern countries, where large number of cases occurs in rice field workers, such as in China and Japan. In France, a monovalent vaccine containing serovar *icterohaemorrhagiae* is licensed for human use. [2]

Newer Leptospiral vaccines are classified into recombinant protein vaccines, lipopolysachharide (LPS) vaccines, inactivated and attenuated vaccines and DNA vaccines.

Recombinant vaccines: They have a great potential against leptospirosis. Recombinant OMP vaccines include OMP L_1, lipoprotein Lip L_{41}, haemolysis associated protein 1 (HAP 1) and immunoglobulin like (Lig) proteins. Loa22 is one of the outer membrane proteins exposed on the cell surface among pathogenic *Leptospira* but not in non-pathogenic *Leptospira*. It has been considered as a candidate for a novel vaccine. Recombinant lipoprotein vaccines include Lip L_{32}, Lip L_{45} and Lip L_{21} targets. Recombinant virulence factor vaccines are directed against Leptospiral virulence factors including Fla A, Fla B, Hsp 58, Sph H and Chp K.

Lipopolysaccharide vaccines: It is shown that LPS vaccines may be serovar independent. But there are reports of few LPS vaccines which are serovar dependent. Therefore, further studies are required to determine whether other LPS vaccines are serovar dependent or independent. If it is independent, it will make LPS vaccines more simple and efficient.

DNA vaccines have several advantages over recombinant protein vaccines. They have simple processing routes, low prices and easy administration properties. Two DNA vaccine trials have been reported. DNA vaccines encoding haemolysis associated protein1 (HAP1) and endoflagellin (Fla B_2) have been developed. [24]

Rodent control:

Rodent control involves keeping surroundings clean and preventing access of rats to the buildings. Rodent control programmes have to be instituted especially during breeding season which synchronizes with monsoon season. Rodent survey should be done during pre monsoon periods and if the live burrows are more than 50/hectare, Zinc phosphide should be used. If it is less than 50/hectare, single dose of Bromadiolone in

cereal baits may be used. Farm houses may be treated with multiple dose anticoagulants Coumatetralyl TP in cereal baits, where non target animals are many in number. [53]

Personal protective measures:

These measures are taken to prevent exposure of cuts to water, wearing footwear and showering promptly after immersion in any dirty water. Chemoprophylaxis with Doxycycline has been shown to be effective in travellers visiting endemic areas. [2]

4. MATERIALS AND METHODS

SOURCE OF SAMPLES:

During the period from January 2012 to July 2013, serum samples were collected from 2362 patients who visited R.L.Jalappa Hospital with fever and were clinically suspected of leptospirosis. The samples were screened for evidence of IgM antibodies for *Leptospira* by a commercially available ELISA kit.

Patients who presented to the hospital with symptoms of fever, myalgia, headache with other constitutional symptoms and cases of pyrexia of unknown origin were included in the study.

METHOD OF COLLECTION OF SAMPLES:

Three ml of plain venous blood sample was collected aseptically and allowed to clot at room temperature. Serum was separated from the blood by centrifugation at 3000rpm for 10mins.

A subset of 250 samples were subjected to IIFA .

An acute serum sample was collected from a total of 250 patients, most often within 7-10 days after the onset of illness. A second, convalescent serum sample to demonstrate four-fold rise in titre of the antibody could be collected from 15 patients after a period of 8-10 days from the date of collection of the first sample. So, 235 serum samples were from patients in acute phase alone and the other 15 samples were paired samples.

PROCESSING OF SAMPLES:

Serum separated in vials was subjected to

↓

A. I_gM ELISA using IVD microlisa kit (J Mitra co.)

B. In house IIFA by using Patoc strain of Leptospira as an antigen

↓

Samples positive by either of the above tests were further tested by

C. MAT by using Patoc strain of Leptospira as an antigen

In case of Paired samples, both the samples were subjected to the tests parallelly.

The details of the above tests are described below.

A. I_gM ELISA

This test was carried out by using a commercial kit, Lepto IgM Microlisa kit which was supplied by J. Mitra Company. According to the manufacturer's performance characteristics, Lepto IgM microlisa has a sensitivity of 97.29% and a specificity of 99.84%.

Figure 13:

IgM ELISA kit by J Mitra co.

Principle: Lepto IgM Microlisa test is an enzyme immunoassay based on the principle of "Indirect ELISA". Recombinant protein mixture of various molecular weights representing immunodominant epitopes are coated onto the microtitre wells as supplied. Serum samples and controls are added to the different microtitre wells and incubated. IgM antibodies to *Leptospira*, if present in the sample, will bind to the specific antigens adsorbed onto the surface of the wells. The plate is then washed to remove the unbound material. Horseradish peroxidise (HRP) conjugated Anti-Human IgM is added to each well. This conjugate will bind *Leptospira* antigen-antibody complex if present. Finally, the substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated. A blue colour will develop in proportion to the amount of *Leptospira* antibodies present in the sample. The colour reaction is stopped by a stop solution. The enzyme substrate reaction is read by EIA reader for absorbance at a wavelength of 450 nm. If the sample does not contain *Leptospira* antibodies, then the enzyme conjugate will not bind and the solution in the wells will be either colourless or only a faint background colour develops.

Test procedure:

The procedure was completed without interruption. The strip holder was fitted with the required number of Lepto Antigen coated strips. All the reagents were dispensed in the centre of the well without touching the wall of the micro well with the tip of the pipette. The sequence of the procedure was carefully followed.

a. Well A-1 was left as substrate blank.

b. 100 µl Negative Control was added in B-1 & C-1 well.

c. 100 µl Positive Control was added in D-1 well.

d. 100 µl of each serum sample, diluted in sample diluents (1:100) was transferred into each well starting from E-1well.

e. Cover seal was applied and incubated at 37ºC for 30 minutes.

g. Working Wash Solution and working conjugate was prepared as specified in the preparation of reagents.

h. Plate was taken out from the incubator after the incubation time was over and, washed 5 times with working Wash Solution.

i. 100 µl of working Conjugate Solution added into each well excluding A-1.

j. Cover seal was applied and incubated at 37ºC for 30 minutes. Repeated the washing step.

m. 100 µl of working substrate solution added into each well including A-1.

n. Incubated at room temperature (20-30ºC) for 30 minutes in dark.

o. 50 µl of stop solution added.

p. Absorbance read at 450 nm within 30 minutes in ELISA READER

Test validity : The following should be within specified acceptance criteria

i) Blank must be < 0.100

ii) Negative Control (NC) or $NC\bar{x}$ Optical density (O.D) must be < 0.3. If it was not so, the run was considered invalid and was repeated.

iii) Positive control (PC) O.D. must be > 1.1. If it was not so, the run was considered invalid and was repeated.

Calculation of results

a. Cut off value = $NC\bar{x} + 0.500$

b. Sample O.D. ratio = Sample O.D ÷ Cut off Value

c. Calculation of Lepto IgM units: Sample O.D. ratio × 10.

Interpretation of results

Lepto IgM units: < 9: Sample - negative for Lepto IgM antibodies.

Between 9 – 11: Sample - equivocal for Lepto IgM antibodies.

> 11: Sample - Positive for Lepto IgM antibodies.

INDIRECT IMMUNOFLUORESCENCE ANTIBODY ASSAY (IIFA) AND MICROSCOPIC AGGLUTINATION TEST (MAT)

For both Indirect Immunofluorescence assay and Microscopic agglutination test, '*Patoc strain' of Leptospira* was used as an antigen, which is a non pathogenic strain obtained from Professor Dr. Dhanapaul, at Government Medical College, Thiruchy.

7 to 8 day old strain was used to coat the slides in IIFA as antigen and directly as an antigen in MAT. So, this strain was maintained in our laboratory by culture as follows:

Maintenance of Patoc strain of Leptospira

Patoc strain of Leptospira was maintained in our laboratory by culturing in 2 types of media.

1. Semisolid medium – Fletcher's medium.

2. Liquid medium – Tryptone Phosphate broth medium.

Figure 14: TPB and Fletcher's medium

Both these media were enriched with addition of Rabbit serum.

Preparation of Fletcher's medium :

Ingredients: Peptone – 0.03gm, Beef extract –0.02gm, Sodium Chloride – 0.05gm, Agar- 0.15gm, Distilled water – 92ml.

Ingredients were transferred into a sterile flask and dissolved well in distilled water. pH was adjusted to 7.2 to 7.4. The medium was heated to boiling point to dissolve. Dispensed at a quantity of 4ml into the sterile screw capped tubes and autoclaved at 121º C for 20 minutes. Cooled to 45º to 50º C and Rabbit serum was added. Rabbit serum was obtained by collecting Rabbit blood as an in house procedure.

Collection of Rabbit blood

Requirements: Rabbits, Sterilized Mac Cartney bottles, sterile blade, Alcohol, Xylene, Cotton.

Procedure: Rabbit was positioned properly. Blood was collected from the ear. Lateral marginal vein in the ear was traced and cleaned with spirit. An oblique nick was made on the anterior wall of the vein. Care was taken not to cut through and through. Blood was collected as drop by drop into the Mac Cartney bottle. Cotton soaked mildly with xylene was used to dissolve the clot if the blood clots. About 30ml was collected at a time from 2 rabbits. Collected blood was refrigerated for 48hrs. Separated serum was transferred to the test tubes, centrifuged and the final supernatant serum was transferred to another sterile Mac Cartney bottle. If not used immediately, it was stored at -20º C until use.

Sterilization of Rabbit serum was done by membrane filtration method. For filtration, filter papers of 2 different pore sizes were used; 0.45µ and 0.2µ obtained from PALL Life sciences. Membrane holder was obtained from Tarson's company. Membrane

filters were placed in the membrane filter holder in such a way that, the 0.45µ was towards the injecting side and 0.2µ was towards the delivering side. Filter papers were assembled in the filter holder and autoclaved at 121º C for 20 minutes before hand. With a 20 ml syringe, Rabbit serum was loaded. It was fitted on one side of the filter holder where it was screwed and the 18G needle was fitted on the delivering side. Serum was dispensed into the media prepared in screw capped bottles under aseptic conditions in a laminar flow near the Bunsen flame. This is represented in Figure 15. Approximately 0.8ml of Rabbit serum which accounts to 30 drops was delivered into 4ml of media (20%). After adding Rabbit serum, heat inactivation was done by placing the tubes in water bath at 56º C for 30 minutes.

Figure 15: Membrane filter holder and Filtering of rabbit serum

Prepared media was subjected to sterility check, to rule out both bacterial and fungal contamination. Tubes were incubated at 37º C for 24hrs to rule out bacterial contamination, and at room temperature for next 48 hrs to rule out fungal contamination. After ensuring sterility, the media was stored in refrigerator at 2 - 8º C.

Preparation of Tryptone Phosphate Broth (TPB) - Liquid medium

Ingredients: Tryptone - 0.135gm, Dextrose – 0.115gm, Sodium Chloride - 0.034gm, Na2HPO4 - 0.017gm, Distilled water – 92ml

Ingredients were transferred in to a sterile flask. Dissolved well in distilled water. pH adjusted to 7.2 to 7.4. Dispensed as 4ml quantity in sterile screw capped tubes. Autoclaved at 121º C for 20 minutes. Cooled to 45 to 50º C. Rabbit serum was added in the same way as described for Fletcher's medium. Sterility checking also was done in the same way. Media were stored in refrigerator at 2 - 8ºC.

Utmost care was taken in the preparation of media, sub culturing and maintenance of the strain to prevent contamination.

Patoc strain of Leptospira was maintained in cultures by Sub culturing once in every 8 days in TPB medium and Sub culturing once in every 15 days in Fletcher's medium. Sub culturing was done aseptically in laminar flow by transferring 300µl of the old culture using sterile micro pipette and tips into the new culture medium. The inoculated culture was kept at room temperature for 8 days in TPB medium and for 15 days in Fletcher's medium. Before sub culturing, the culture was subjected to dark ground microscopy to see whether; there were adequate number of *Leptospira*, there was any contamination, they were healthy and actively motile.

B. INDIRECT IMMUNOFLUORESCENCE ANTIBODY ASSAY (IIFA)

a. Preparation and Pre-treatment of glass slides:

Using the diamond/glass marking pencil, 4 small circular areas measuring approximately 0.5 mm were made on the glass slides. Slides were boiled in distilled water for about 10-15 minutes after which they were washed with detergent and hot water thoroughly. Then, the slides were dried and stored by wrapping in tissue paper.

b. Coating the pre-treated slides with antigen:

A 7-8 day old culture of leptospira in TPB medium was taken

↓

The culture tube was centrifuged at 2500 rpm for 5 mins

↓

Supernatant was transferred to a sterile vial

↓

To the sediment, 300 µl of TPB medium is added and mixed well

↓

One loopful of prepared antigen was spotted on the circles over the glass slides and air dried

↓

One of the slides was subjected to Fontana's staining* to confirm coating of the organism

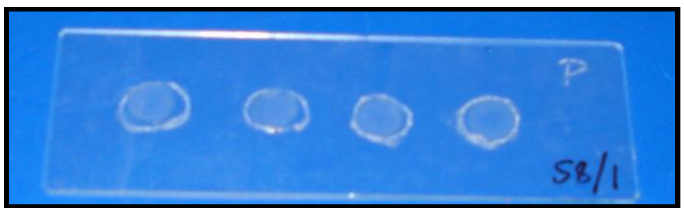
↓

Acetone fixation was done by immersing the slides in coplin jar with acetone at -20ºc for 20 minutes

↓

Once prepared, such slides were stored at +4 ºc for up to 3 months. The antigen coated slide is shown in Figure 16.

Figure 16: Antigen coated slide



***** Fontana's staining technique: [78]

Objective: - To ensure that the slide coated with Patoc strain of leptospira, retains coating Staining reagents: 1. Fixative; Glacial acetic acid-1 ml, 40% Formalin-2ml, Distilled H_2o-100ml. 2. Mordant: Phenol – 1gm, Tannic acid – 5gm, Distilled H_2o-100ml. 3. Ammoniacal Silver nitrate.

Procedure:

- Smear was treated for 3 times, 30 seconds each time with the fixative.
- Fixative was washed off with absolute alcohol and allowed to act for 3 minutes.
- Excess alcohol was drained and carefully burnt off until the film was dry.
- Mordant was poured, heated till fumes arose and allowed to act for 30 seconds

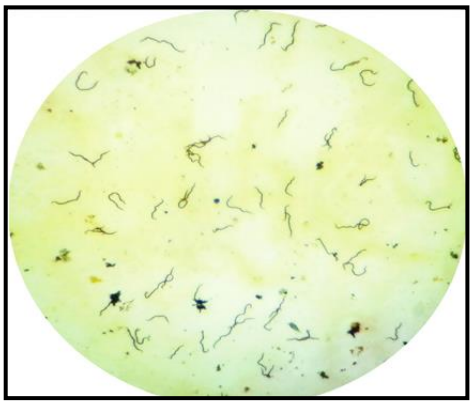
-Washed well in distilled water and the slides were dried

-Treated with ammoniacal silver nitrate solution. Heated till fumes arose and allowed to act for 30 seconds or till the film became brown in colour

Washed with distilled water, dried and mounted with a DPX mounting solution

-Focussed under low power, then under oil immersion objective.

Figure 17: Fontana' staining



Leptospires were seen as brownish black spiral organisms against a yellowish brown Background as shown in Figure 17.

Principle: Indirect Immunofluorescence is a serological test to demonstrate antibodies in the sera using specific antigen-antibody reactivity in the presence of fluorescent dyes. The antibodies present in the test sample which bind to the antigens, are visualized by a fluorescent labelled anti antibodies directed at the antibodies.

c. IIFA Procedure

Serial dilutions of test sera + antigen coated on the slide

↓

Incubated at 37°c x 30 minutes and Washed

↓

Fluorescein isothyocyanate conjugated goat anti-human IgG is added

↓

Incubated at 37°c x 30 minutes and Washed

↓

Examined under a fluorescent microscope for visible fluorescence

↓

End point titre - taken as the highest dilution of serum giving a visible fluorescence

Requirements:

Antigen coated slides, Test serum, Micropipette and tips, Phosphate buffered saline (PBS), FITC conjugate, Coplin jars, Glycerol-phosphate buffer mounting solution, Fluorescent microscope

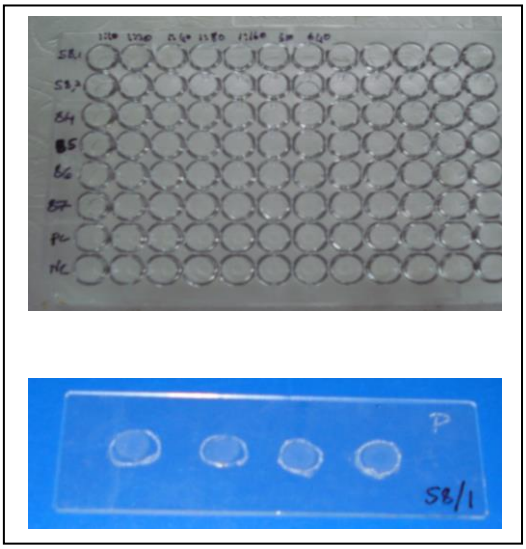
Procedure:

- Slides were brought to the room temperature.
- Paired samples were tested simultaneously.
- Serum sample number and dilutions were marked properly on the Sterile microtitre plate

-Test serum was serially diluted in the wells of microtitre plate by doubling dilution

method using PBS* to achieve dilutions of 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640, as shown in the Figure 18.

Figure 18: Microtitre plate, Antigen coated slide and FITC conjugate.



-10µl each of different serum dilutions were added on to different antigen spots on a single slide. So, one slide was used to test 4 different dilutions of a single serum sample at a time.

- Slides were placed in a moist, closed container and incubated at 37°c for 30 minutes.

-Washing was done by immersing the slides in a coplin jar containing PBS. The slides were allowed to stand for 10 minutes each time for 3 times. Finally, they were rinsed with distilled water and air dried.

- 10 µl of 1:8 diluted goat anti human IgG FITC conjugate (1:8 diluted)** was added to each of the spot areas.

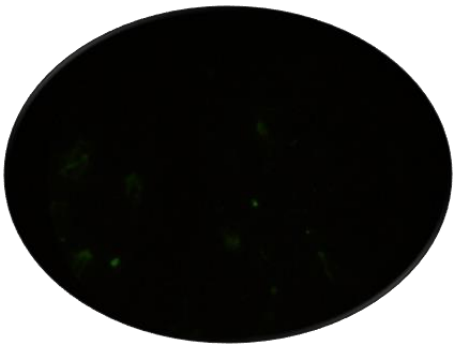
- Again the slides were placed in a moist, closed container and incubated at room temperature in dark.

- Washing, rinsing and drying were repeated as described above but in dark.

- A drop of Glycerol-phosphate buffer mounting solution was placed over the circular spot areas and covered with a cover slip. This was to avoid non specific fluorescence.

- The slide was focussed under oil immersion objective in fluorescent microscope and looked for the yellowish green fluorescence of the intact *Leptospira*. This is shown in Figure 19.

Figure 19: Yellowish green fluorescence of intact *Leptospira*



*PBS : Sodium chloride – 8gm, Potassium chloride – 0.2 gm, Disodium hydrogen phosphate –1.44 gm, Potassium dihydrogen phosphate – 0.2 gm, Distilled water – 1000ml. pH – 7.2

**Goat antihuman IgG FITC conjugate was obtained from Bangalore Genei, Xeno Bio Solutions, Bangalore. Optimal conjugate dilution was standardized as 1:8 by checkerboard titration as follows: Checkerboard titration was achieved by testing a series of doubling dilutions of FITC conjugate on the series of doubling dilutions of known positive and negative serum samples and looking for the optimal fluorescence seen.

Interpretation of the results: [79]

The intensity of the fluorescence was read and graded as follows

++++: brilliant fluorescence, +++: bright fluorescence, ++: clearly visible fluorescence +: just visible fluorescence +/-: weak fluorescence

The titre of the serum was taken as the highest dilution of the serum giving 1+ fluorescence. It was characteristic for a positive serum that the intensity of the fluorescence diminished gradually in the higher dilutions.

To estimate the cut off titre in IIFA, to interpret as positive or negative, the geometric mean titre was calculated for all the positive as well as negative samples. The Geometric mean titre (GMT) was calculated as follows. The titres were converted in to logarithmic values and were summated. This was divided by the number of samples and the final value was derived. Antilog was calculated for this value which is the geometric mean titre. [80]

C. MICROSCOPIC AGGLUTINATION TEST: (MAT) [7]

Requirements: Round bottomed microtitre plate, *Leptospira* in TPB medium (antigen), Test serum, positive control serum (Rabbit hyper immune serum), Normal saline (NS), Micropipette with sterile tips, Thin glass slides, Cover slips, Dark field microscope (DFM)

Principle: Based on the antigen-antibody reaction which agglutinates and forms microscopically visible clumps and reduction in the number of Leptospires.

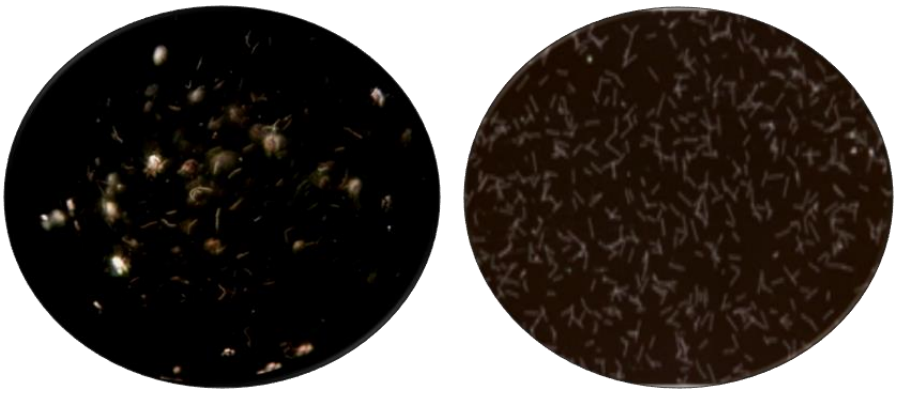
Procedure:

-The culture of *Leptospira* in TPB medium was brought to the room temperature.

-A drop of the liquid culture was placed on the slide, covered with a cover slip and examined under DFM. Number of Leptospires had to be approximately 200/ high

power field. This was counted approximately by four quadrant technique. If the number of leptospires was >200/HPF, the culture was diluted with approximate amount of normal saline to make it up to 200/HPF. If the number of leptospires was <200/HPF, the culture was not used for the test. This is shown in Figure 20.

Figure 20: Dark field microscopic picture of *Leptospira* in culture



< 200/HPF 200/HPF

-A sterile 96 well microtitre plate was taken. Serum samples to be tested were arranged and proper labelling on the microtitre plate was done in each row.

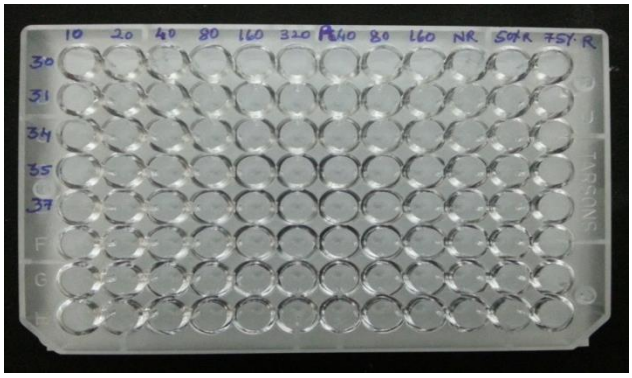
-In the first row, last 3 wells (wells 10,11 and 12) were chosen to prepare the controls for the reduction in the number of leptospires as shown below

No reduction : 25µl antigen + 25 µl NS.
50% reduction: 25µl antigen + 75 µl NS
75% reduction: 25µl antigen + 175 µl NS
} Mixed well

-The wells 7, 8 and 9 were chosen for positive control. Rabbit hyperimmune serum* was used as the positive control serum. 1:40, 1:80 and 1:160 dilutions of rabbit hyperimmune serum were done in wells 7, 8 and 9 respectively.

-6 wells in each row were used for one serum sample. This is shown in Figure 21.

Figure 21: Microtitre plate with serum dilutions in MAT



-Well 1: Master dilution was done – 90 µl NS + 10 µl test serum. Mixed well. This accounts to a dilution of 1:10.

-Dispensed 50 µl of NS into the subsequent 5 wells.

-From well no.1, 50 µl was transferred into well 2. It was mixed well and again 50 µl was transferred into well 3.

-The same procedure was repeated in the subsequent wells till the 6th well and the last 50 µl was discarded, achieving serum dilutions of 1:10, 1:20, 1:40, 1:80, 1:160 and 1:320 in wells 1,2,3,4,5, and 6 respectively.

-5o µl antigen was added to the wells 1-6. Also 5o µl antigen was added to the wells 7, 8 and 9 which are positive control wells.

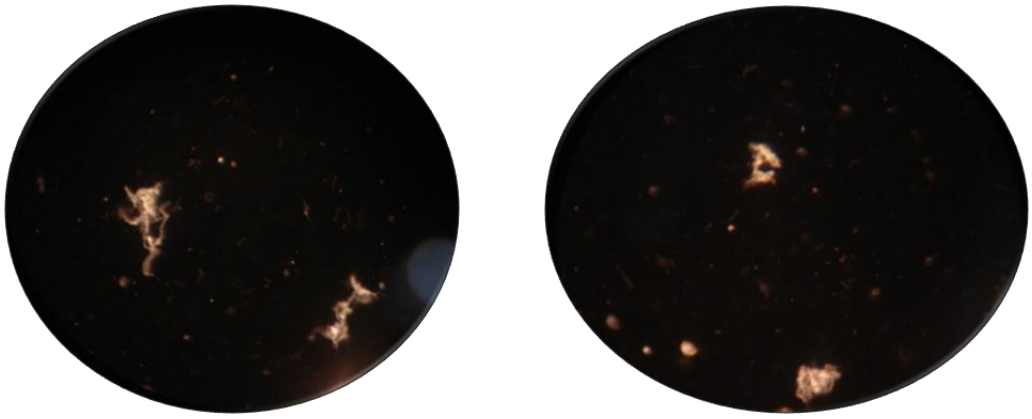
-The final dilutions achieved were 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640 in wells 1, 2, 3, 4, 5, and 6 respectively.

-The plate was tapped to mix. It was covered with aluminium foil and incubated in moisture at 37ºc for 2 hours.

-After 2 hours, wet mount from each dilution of the positive control serum was prepared and visualized under DFM for formation of clumps and ≥50%reduction in the number of Leptospires by comparing with the controls in wells 10, 11 and 12.

-Wet mount from each serum dilution was screened for the agglutination and reduction in the number of Leptospires. This is shown in the Figure 22.

Figure 22: Agglutination of Leptospires in MAT



-According to the standard guidelines, the antibody titre was taken as the highest dilution of serum which showed agglutination and ≥50%reduction in the number of *Leptospira*.

Demographic & Clinical Data

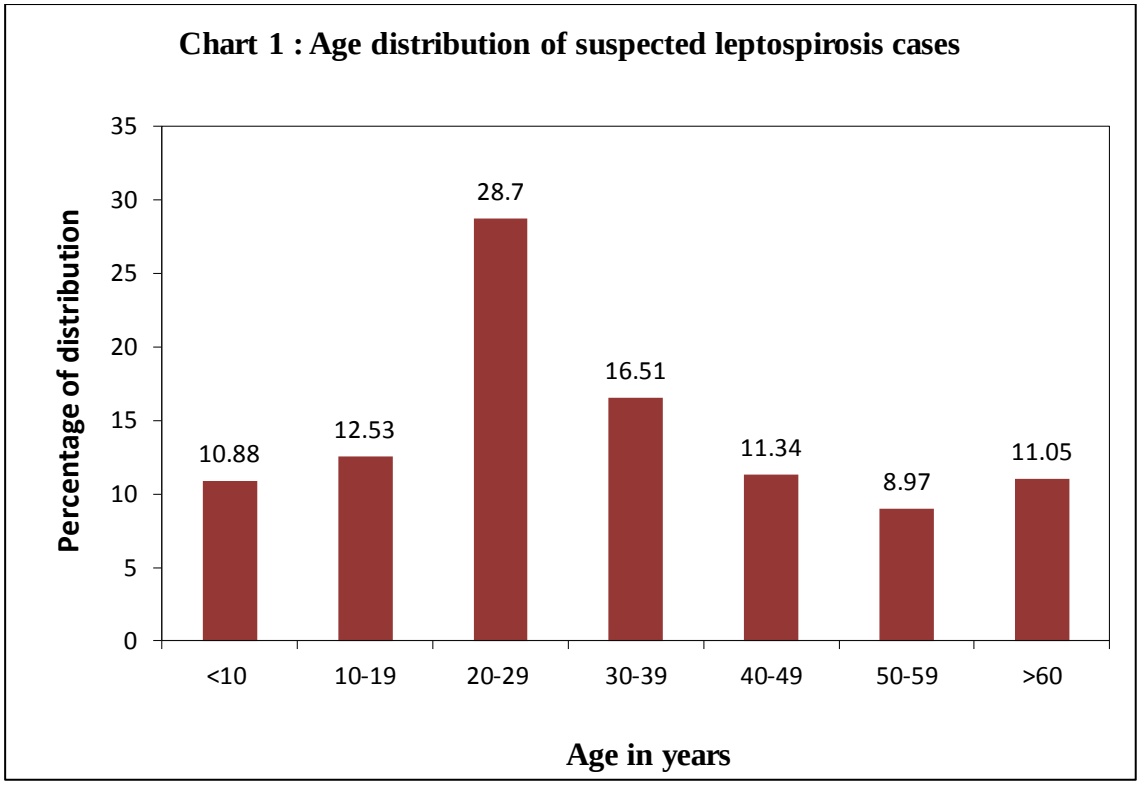
The demographic data was collected for all the 250 patients. Clinical data of the patients who were tested positive by IgM ELISA test was collected.

5. RESULTS

During the period from January 2012 to July 2013, serum samples collected from 2362 patients, who had fever and were clinically suspected of leptospirosis, were screened for evidence of IgM antibodies for *Leptospira* by a commercially available ELISA kit which was based on the principle of indirect ELISA. Among those patients with fever who were tested, children accounted for 527 (22.32%) cases tested and the rest [1835 (77.68%)] were adults. The age distribution is presented in Table 9 and Chart 1.

Table: 9 – Age distribution of suspected leptospirosis cases

Age(years)	No. screened (%)
<10	257 (10.88)
10-19	296 (12.53)
20-29	678 (28.7)
30-39	390 (16.51)
40-49	268 (11.34)
50-59	212 (8.97)
>60	261 (11.05)
Total	2362



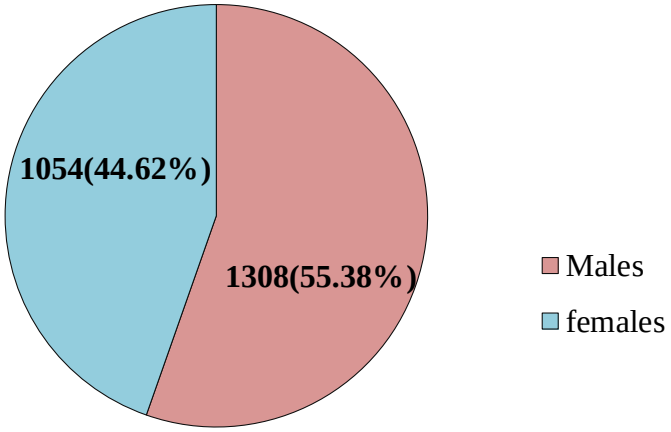
The patients screened for evidence of leptospirosis as seen in the diagram belong to all the age groups. The youngest patient screened was a child of 26 days and the oldest patient screened was of 90 years age. Adults between the age group 20-29 accounted for 28.7%, the highest number of patients tested. This preponderance of samples from patients in this age group is statistically significant (P value <0.001).

Among 2362 patients, 1308 (55.38%) were male patients and 1054 (44.62%) were female patients. The sex distribution is shown in Table 10 and depicted in Chart 2.

Table 10: Gender distribution of suspected leptospirosis cases

Gender	Number (%)
Males	1308 (55.38)
Females	1054 (44.62)
Total	2362

Chart 2: Gender distribution of suspected leptospirosis cases



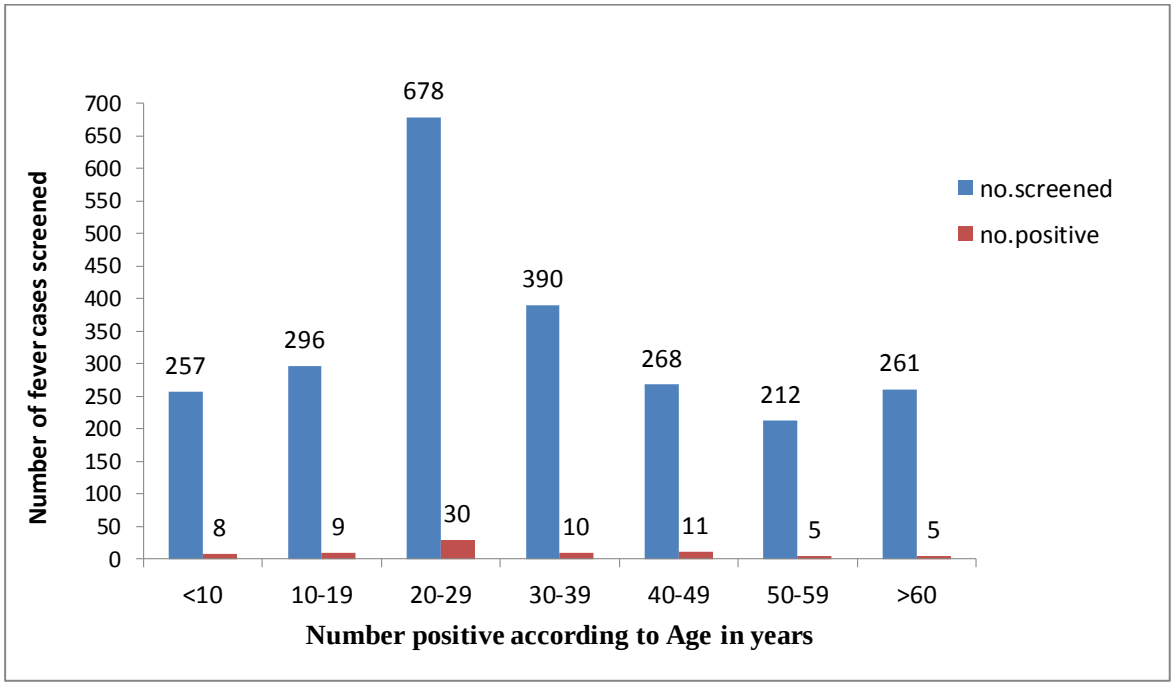
Of the 2362 patients, who were screened, 78 (3.3%) patients were positive for IgM class of antibodies for leptospira in their serum, and the rest [2284 (96.7%)] were negative for IgM class of antibodies in their serum. This accounts to a overall seropositivity rate of 3.3% for leptospirosis in patients with fever who visited our hospital during a period of 1year and 7 months.

The positivity of IgM ELISA test in each age group according to the number of suspected cases of leptospirosis in that particular age group is shown in Table 11 and Chart 3.

Table11: Age wise distribution of IgM ELISA Positivity in suspected leptospirosis cases

Age(years)	No. of suspected leptospirosis cases screened	No. positive (%)
<10	257	08 (3.11)
10-19	296	09 (3.04)
20-29	678	30 (4.42)
30-39	390	10 (2.56)
40-49	268	11 (4.10)
50-59	212	05 (2.35)
>60	261	05 (1.91)
Total	2362	78

Chart 3: Age wise distribution of IgM ELISA Positivity in suspected leptospirosis cases

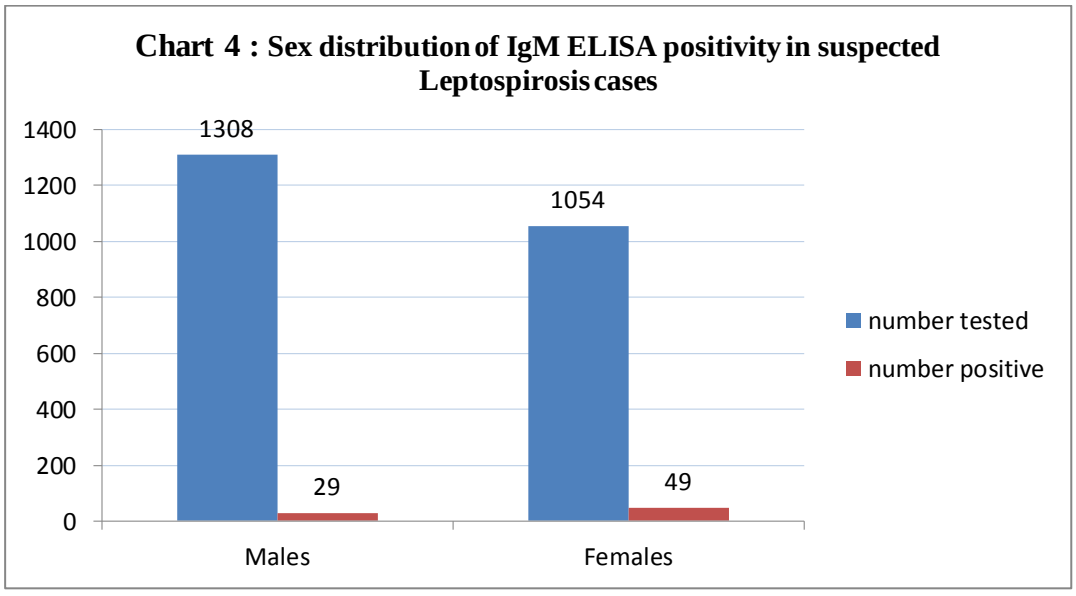


From the above data, one can make out that there is no difference in serological positivity for leptospirosis in the different age groups.

The positivity of IgM ELISA test in both the sexes according to the total number tested is presented in Table 12 and Chart 4, which shows that there is no difference in serological positivity for leptospirosis in both the sexes.

Table 12: Sex distribution of IgM ELISA positivity in suspected Leptospirosis cases

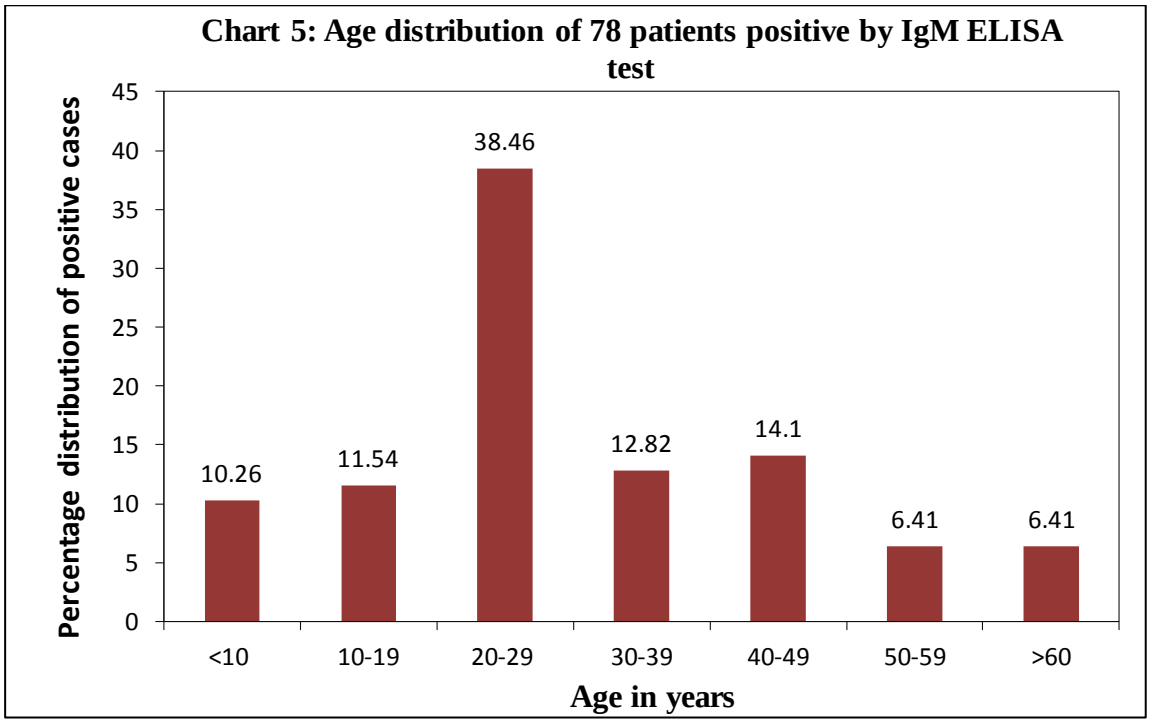
Sex	Number screened	Number positive (%)
Males	1308	29 (2.22)
Females	1054	49 (4.65)
Total	2362	78



The age wise distribution of positivity among 78 patients who were positive for IgM class of antibodies is presented in Table 13 and Chart 5.

Table 13: Age distribution of 78 patients positive by IgM ELISA test

Age(years)	Number positives (%)
<10	08 (10.26)
10-19	09 (11.54)
20-29	30 (38.46)
30-39	10 (12.82)
40-49	11 (14.1)
50-59	05 (6.41)
>60	05 (6.41)
Total	78

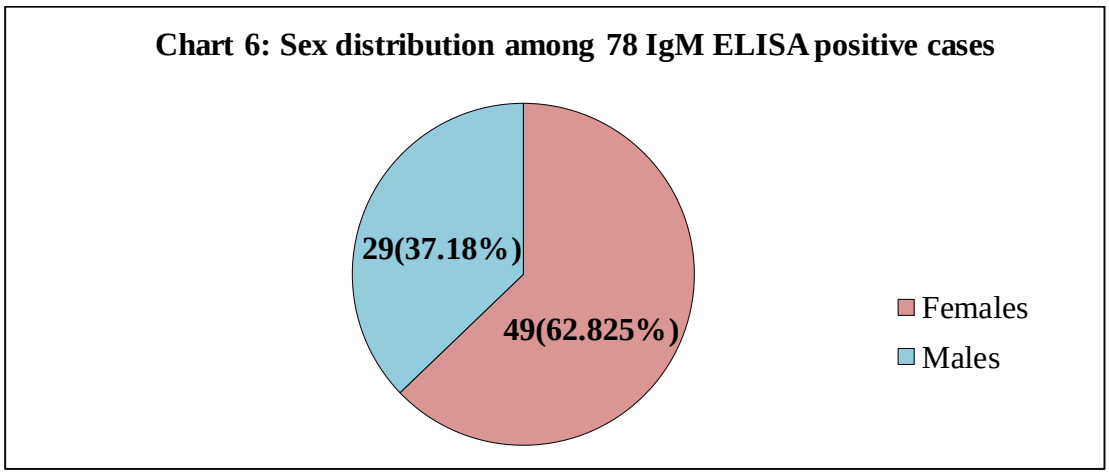


Among 78 patients positive for IgM class of antibodies, 30 (38.46%) patients belonged to 20-29 year age group where as only 5 (6.41%) patients each belonged to 50-59 and >60 years age group. The rest of the age groups accounted for 10-14% of the patients in each age group. This finding of higher percentage of distribution in 20-29 year age group is highly significant as the 'P' value, as calculated by Chi-square test is <0.001. As a significantly larger proportion of patients with fever presented in 20-29 year age group, the number of sera positive for IgM antibodies for *Leptospira* among all the cases is highest in this age group. Thus, leptospirosis appears to be a serologically confirmed disease, significantly in this age group.

The distribution of positivity in males and females among these 78 patients is presented in Table 14 and Chart 6.

Table 14: Sex distribution among 78 IgM ELISA positive cases

Gender	Number positives (%)
Females	49 (62.82)
Males	29 (37.18)
Total	78

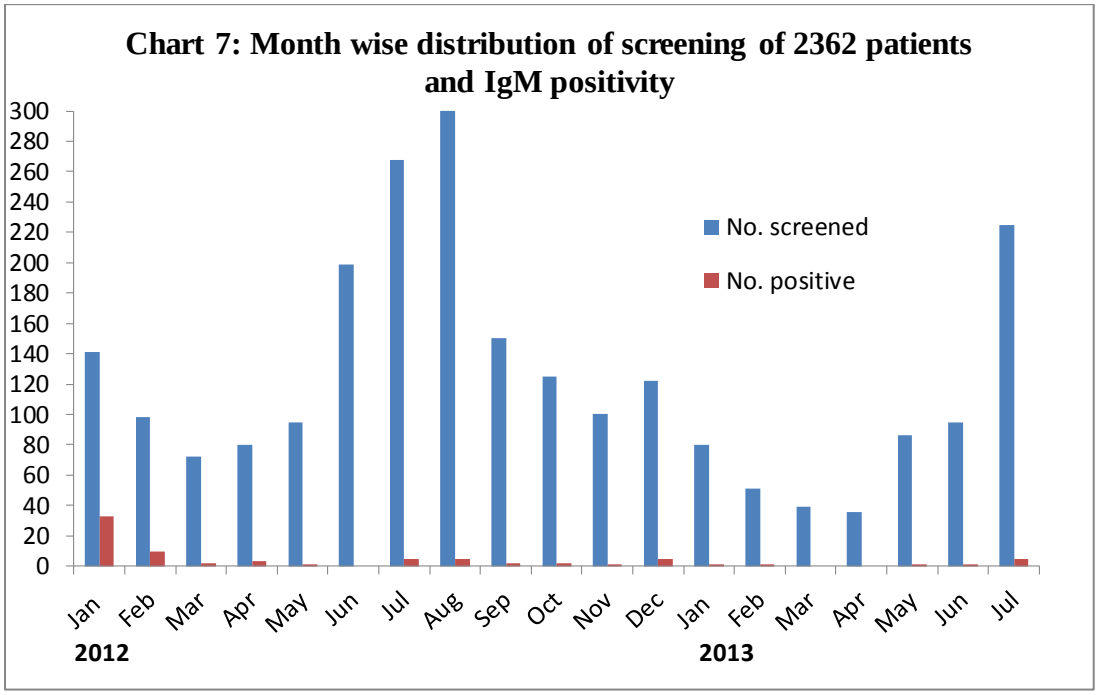


This finding of higher percentage of distribution among females is statistically significant as 'P' value is <0.001.

Month wise distribution of screening of 2362 patients and IgM positivity according to the numbers tested for a period of 1 year and 7 months is presented in Table 15 and Chart 7.

Table 15: Month wise distribution of screening of 2362 patients and IgM positivity rate

2012	Month	No. of suspected leptospirosis cases screened	No. of positives(%)
	January	141	33 (23.4)
	February	98	10 (10.2)
	March	72	02 (2.77)
	April	80	03 (3.75)
	May	95	01 (1.05)
	June	199	00 (00)
	July	268	05 (1.86)
	August	300	05 (1.66)
	September	150	02 (1.33)
	October	125	02 (1.6)
	November	100	01 (1)
	December	122	05 (4.1)
2013	January	80	01 (1.25)
	February	51	01 (1.96)
	March	39	00 (00)
	April	36	00 (00)
	May	86	01 (1.16)
	June	95	01 (1.05)
	July	225	05 (2.22)
Total	1 year 7 months	2362	78 (3.3)

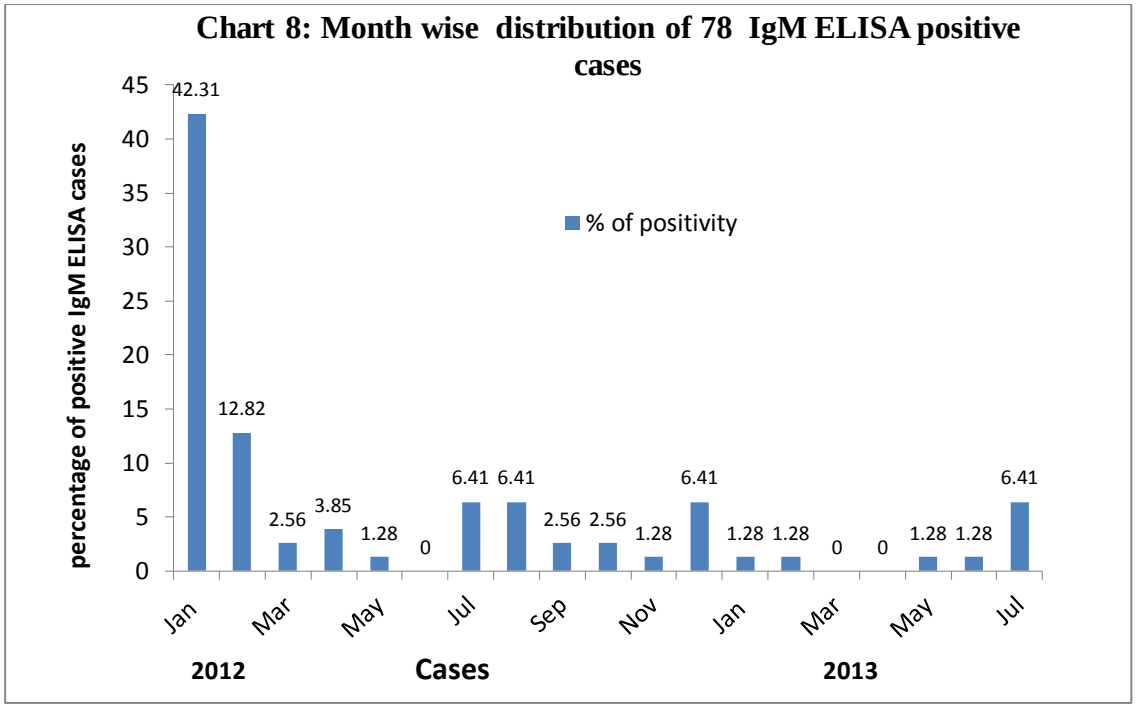


Out of the 141 cases tested in January 2012, 23.4% were positive for IgM class of antibodies to Leptospirosis. This was followed by 10.2% in the month of February. But in all the other months, the percentage of seropositivity was low. In addition, there were months in which, even though considerable number of patients were tested, there was no positivity.

The monthwise distribution of 78 IgM ELISA positive cases is presented in Table 16 and depicted in the Chart 8.

Table 16: Month wise distribution of 78 IgM ELISA positive cases

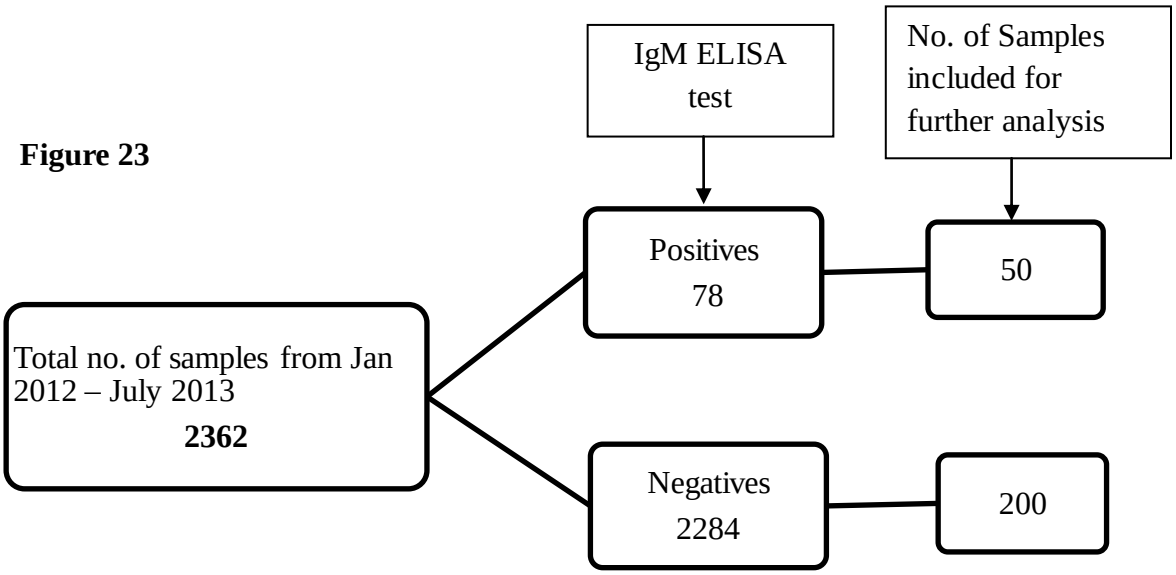
2012	Month	No. of positives(%)
	January	33 (42.31)
	February	10 (12.82)
	March	02 (2.56)
	April	03 (3.85)
	May	01 (1.28)
	June	00 (00)
	July	05 (6.41)
	August	05 (6.41)
	September	02 (2.56)
	October	02 (2.56)
	November	01 (1.28)
	December	05 (6.41)
2013	January	01 (1.28)
	February	01 (1.28)
	March	00 (00)
	April	00 (00)
	May	01 (1.28)
	June	01 (1.28)
	July	05 (6.41)
Total	1 year 7 months	78

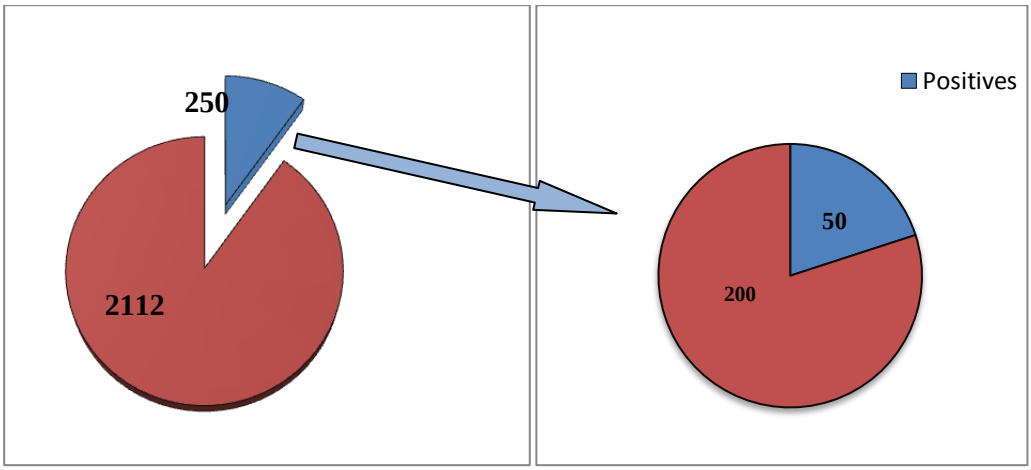


This data also shows that maximum number of cases were in January 2012 accounting to 44.3% of the total 78 positive cases by IgM ELISA followed by 12.82% cases in February 2012.

Out of the 2,362 serum samples from the patients, 250 serum samples were randomly chosen for the study, for further analysis by the other two serological tests; IIFA and MAT. This is represented in Figure 23.

Figure 23





The clinical features of 50 cases included in the study which were positive by IgM ELISA are presented in Table 17.

Table 17: Clinical features of 50 positive cases

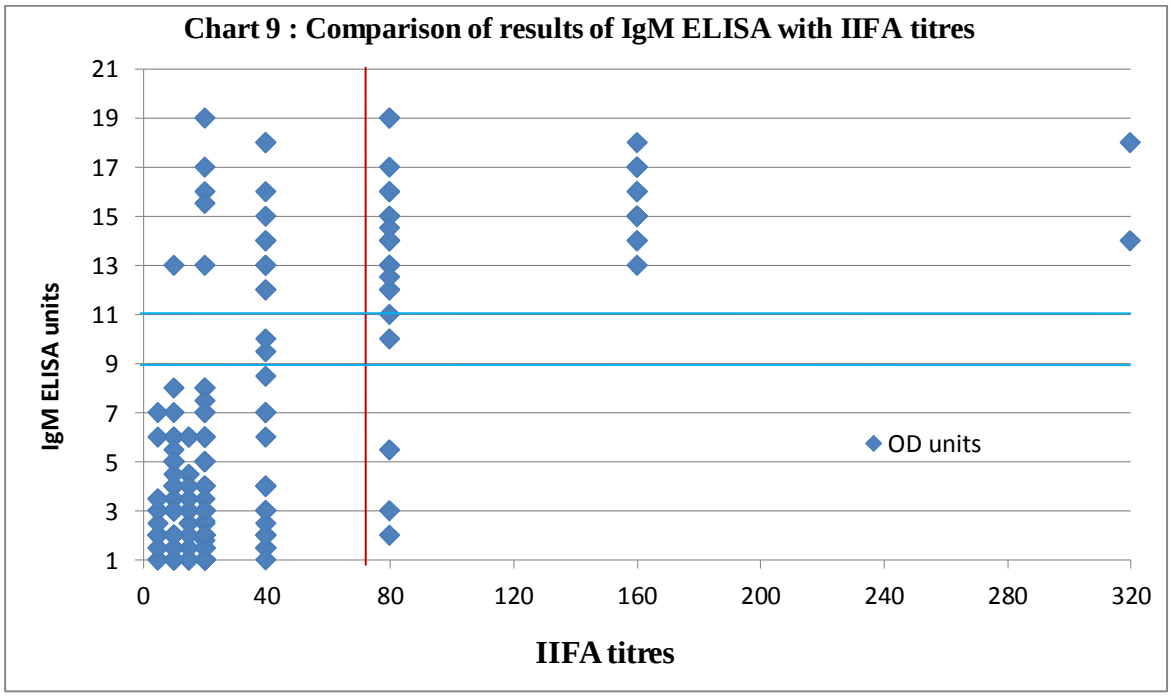
Clinical features	**Adults (n=41) No. (%)**	**Children (n=9) No. (%)**	**Total (n=50) No. (%)**
Fever	41 (100)	9 (100)	50 (100)
Myalgia	32 (78)	4 (44.44)	36 (72)
Head ache	28 (68.3)	4 (44.44)	32 (64)
Conjunctival suffusion	21 (51.21)	3 (33.33)	24 (48)
Vomiting	13 (31.7)	6 (66.66)	19 (38)
Cough/ Breathlessness	15/4 (36.6/ 9.75)	3/1 (33.33/ 11.1)	18/5 (36/10)
Purpura/ Ecchymosis	05 (12.2)	1 (11.1)	06 (12)
Jaundice	03 (7.31)	0 (0)	03 (6)
Hepatomegaly	11 (26.82)	3 (33.33)	14 (28)
Splenomegaly	06 (14.63)	2 (22.22)	09 (16)
Altered sensorium	01 (2.44)	1 (11.1)	02 (4)
Laboratory parameters			
Albuminuria	10 (24.4)	2 (22.22)	12 (24)
Chest X-Ray showing features of pneumonia	06 (59.65)	2 (22.22)	08 (16)
Leucocytosis/leucopenia	14/6 (34.14/14.63)	5/0 (55.55/0)	19/6 (38/12)
Thrombocytopenia	11 (26.82)	3 (33.33)	16 (28)

Six clinical features were commonly seen in these 50 patients. They were fever (100%), myalgia (72%), headache (64%), conjunctival suffusion (48%), vomiting (38%), cough/breathlessness (36/10%) and leucocytosis (38%). Jaundice was present only in three patients. Vomiting was present more in children than in adults. All the 50 patients except two patients met a score of >25 in the modified Faine's criteria. These 48 patients can be taken as presumptive cases of Leptospirosis clinically. The other two patients who had only fever and headache/ myalgia as the clinical manifestation, scored only 19 as per modified Faine's criteria which suggests that they are not cases of Leptospirosis. All the patients who were admitted were treated with either IV Crystalline Penicillin/ Ceftriaxone. A few were treated with oral doxycycline on an outpatient basis. Forty eight patients (96%) recovered. The other two patients expired accounting to a mortality rate of 4%. One of these two patients, a 60 year old woman expired due to Acute respiratory distress syndrome (ARDS) and the other who was a 30 year old woman expired due to multiple organ dysfunction syndrome (MODS). Both these patients did not have any co-morbid condition.

IIFA test results performed on the 250 serum samples is presented in Table 3. IIFA antibody titres of 250 samples in comparison with ELISA are presented in Table 18 and Chart 9.

Table 18: Comparison of IgM ELISA Results with IIFA titres (total n=250)

Serological test		IgM ELISA positive (n=50)	IgM ELISA negative (n=200)
IIFA titers	<1:20	1 (2%)	86 (43%)
	1:20	5 (10%)	85 (42.5%)
	1:40	10 (20%)	25(12.5%)
	1:80	18 (36%)	4 (2%)
	1:160	14(28%)	00
	1:320	2(4%)	00



Cut off titre in IIFA – 80 Cut off value in IgM ELISA

Cases showing a particular titre. *Note*: one such indicates 1 and more than 1 sample.

To interpret the results of IIFA as positive or negative, the titre was calculated as mentioned earlier in material and methods. This was arrived at, by estimating the geometric mean titre value of IIFA titres in those patients who were tested negative for IgM class of antibodies by ELISA test considering them as a part of general population.

Estimation of Geometric mean titre in negative samples

Log $_{\text{IIFA titre}}$ = x × f (no. of samples) = fx

Log_{10} = 1.00 x 86 = 86

Log_{20} = 1.30 x 85 = 110.5

Log_{40} = 1.60 x 25 = 40

Log_{80} = 1.90 x 04 = 7.6

Log_{160} =2.20 x 00 = 00

Log_{320} =2.50 x 00 = 00

Σ fx = 244.1 ÷ 200 = 1.22 | Antilog of 1.22 = 16.6

The 3 standard deviation of 16.6 on the positive side accounts to a titre of 26.17. Therefore, a titre of 1:40 should be actually taken as a cut off titre in IIFA test. However, as there is no difference between subsequent two titres in serology and also as others in the earlier studies have taken 1:80 as the cut off titre, we also have taken 1:80 as the cut off titre to make the results more accurate.

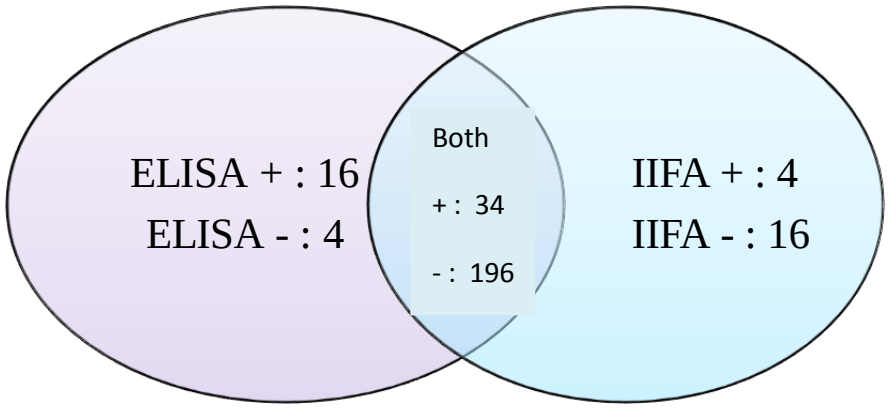
Among 50 samples which were positive in IgM ELISA test, 34 (68%) showed titres of ≥80 in IIFA, the Geometric mean titre being 74. The Geometric mean titre of negative ELISA samples is 16. The Geometric mean titres in IIFA test of IgM positives was about 5 folds more than IgM negative sera.

The comparison of IIFA results with respect to IgM ELISA is shown in the Table 19 and Chart 10.

Table 19: Comparison of results of IgM ELISA and IIFA

Serological test	IIFA positive	IIFA negative	Total
IgM ELISA positive	34	16	50
IgM ELISA negative	04	196	200
Total	38	212	250

Chart 10: Results of ELISA and IIFA on 250 samples



IIFA was positive in 38 (15.2%) samples and negative in 212 (84.8%) of the 250 serum samples. IIFA was positive only in 16 (47.05%) of the 34 IgM ELISA positive cases. Four cases which were negative by IgM ELISA were positive by IIFA. One hundred and ninety six cases were negative in both the tests. This accounts to a

concordance of 92% between IgM ELISA and IIFA. Concordance (percentage of agreement) [81] is calculated as follows:

Concordance of tests 1 and 2:

$$= \frac{a+d}{a+b+c+d} = \frac{196+34}{196+4+16+34}$$

$$= \frac{230}{250}$$

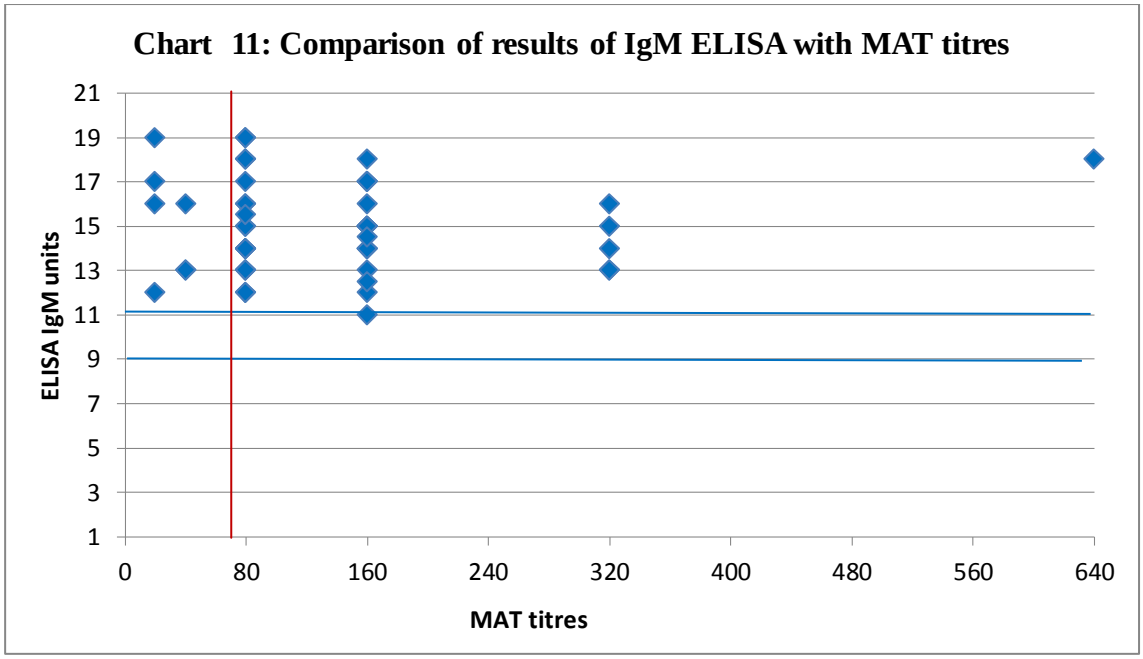
$$= 92\%$$

Serological test		Test 1 (IIFA)	
		_	+
Test 2 (IgM ELISA)	_	a (196)	b (4)
	+	c (16)	d (34)

MAT was performed as a confirmatory test on those 54 samples which were either positive by IgM ELISA or by IIFA. The result of MAT in IgM ELISA positive cases is presented in Table 20 and Chart 11.

Table 20: Comparison of results of IgM ELISA with MAT titres

Serological test		IgM ELISA positive (n=50)
MAT titres	1:20	4(8%)
	1:40	3(6%)
	1:80	19(38%)
	1:160	18(36%)
	1:320	5 (10%)
	1:640	1 (2%)



Cut off titre in MAT – 80, Cut off value in IgM ELISA

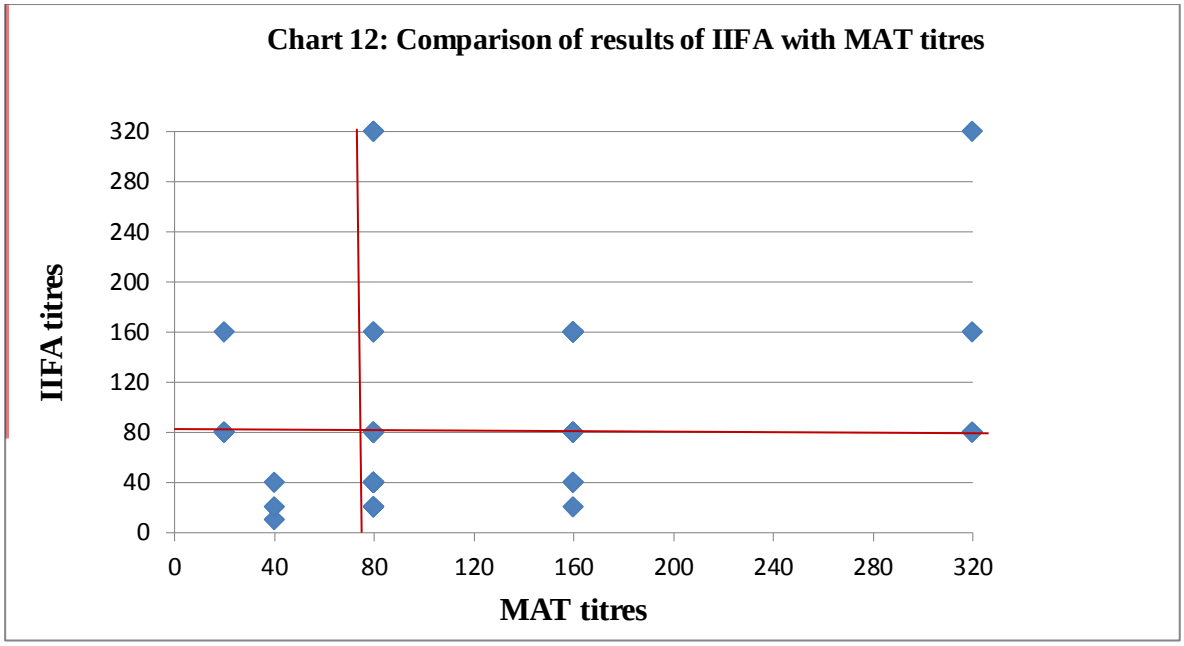
Cases showing a particular titre. *Note*: one such may indicate more than 1 sample.

The cut off titre for MAT was fixed as 1:80, as recommended by other studies. MAT was positive in 43 of the 50 IgM ELISA positive cases accounting to 86% of the cases.

Comparison of MAT titres with IIFA is shown in the Table 21 and Chart 12.

Table 21: MAT titres in IIFA positive cases (n=38)

Serological test		IIFA Positive cases (n=38)
MAT titres	1:20	4(10.52%)
	1:40	3(7.9%)
	1:80	11(28.95%)
	1:160	15(39.47%)
	1:320	4(10.52%)
	1:640	1 (2.63%)

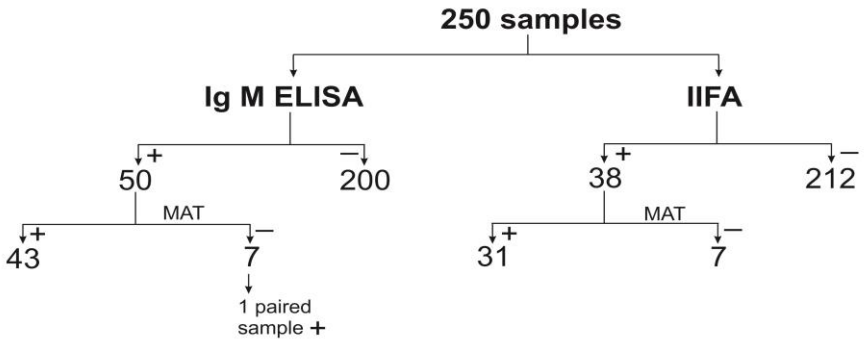


: Cut off titre

: Cases showing particular titre. *Note*: one such may indicate more than 1 sample.

MAT was positive in 81.57% of the 38 cases that were positive by IIFA and was negative in the rest.

On a whole, MAT was positive in 44 (81.48%) out of 54 samples and was negative in 10(18.51%) samples. MAT which was done on 4 cases which were negative by IgM ELISA but positive in IIFA, showed one case as positive.



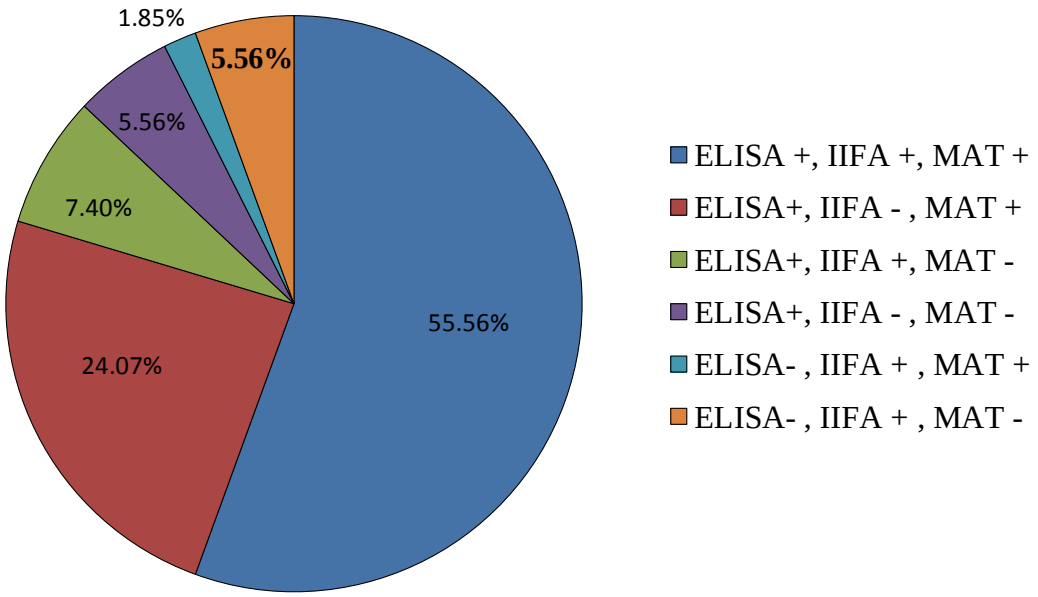
On a whole MAT + cases: 44/54 cases

The results of 54 serum samples which were positive by any of the 3 tests is shown in Table 22 and depicted in the Chart 13.

Table 22: Results of Seropositive leptospirosis cases (n=54)

Results	Number (%)
ELISA +, IIFA +, MAT +	30 (55.56)
ELISA+, IIFA - , MAT +	13 (24.07)
ELISA+, IIFA +, MAT -	04 (7.40)
ELISA+, IIFA - , MAT -	03 (5.56)
ELISA- , IIFA + , MAT +	01 (1.85)
ELISA- , IIFA + , MAT -	03 (5.56)
Total	54

Chart 13: Comparison of positivity in ELISA, IIFA and MAT (n=54)



Out of the 54 samples, 30 (55.56%) were positive in all the 3 tests. Thirteen (24.07%) were positive only in ELISA and MAT test while they were negative in IIFA test. Four (7.40%) samples were positive in both ELISA and IIFA but were negative in MAT test. ELISA alone was positive in 3 samples (5.56%). Four cases which were negative by ELISA but positive by IIFA were also subjected to MAT test and among them, only 1(1.85%) was positive by MAT, where as the other 3 (5.56%) were negative.

This comparison shows that the congruity between all the 3 tests is 55.56%. Considering at least two of the three serological tests which are positive for a sample as the cases of leptospirosis, 48 cases (88.88%) out of 54 cases can be considered as confirmatory cases of leptospirosis.

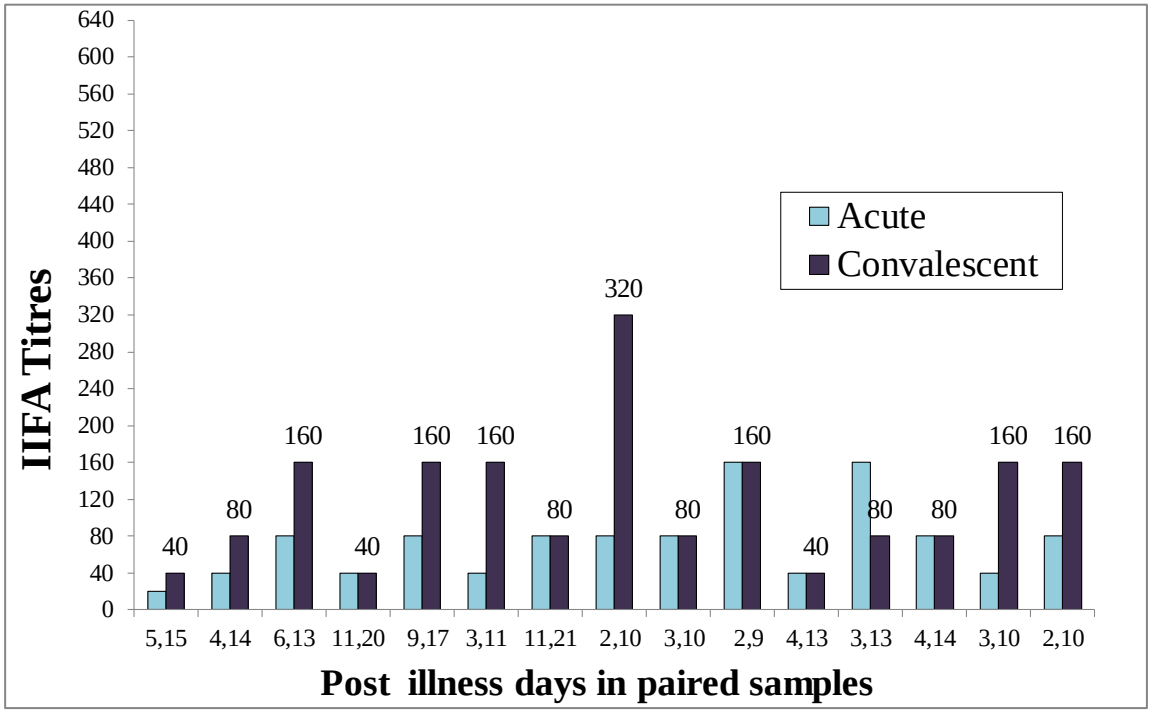
IgM ELISA alone positive in 4(7%) of the cases as well as IIFA alone positive in 3(5.26%) cases can be considered as truly false positive cases. Considering MAT as a reference standard test, false positivity rate of ELISA is found to be 12% and false positivity rate of IIFA is found to be 18.8%.

Paired samples were obtained from 15 patients during the study. They were subjected to IIFA and MAT simultaneously. The results on 15 Acute-convalescent paired sera of patients suspected of Leptospirosis are presented in Tables 23 and 24 and depicted in the Charts 14 and 15.

Table 23: Results of IIFA test on Acute – Convalescent Paired samples (n=15)

Sl no.	Specimen no.	Days PI	IIFA		Interpretation
			Acute	Convalescent	
1	767200 -1 -2	5 15	1:20	1:40	No 4 fold rise in titres
2	767733 -1 -2	4 14	1:40	1:80	No 4 fold rise in titres
3	767810 -1 -2	6 13	1:80	1:160	No 4 fold rise in titres
4	767839 -1 -2	11 20	1:40	1:40	No 4 fold rise in titres
5	767944 -1 -2	9 17	1:80	1:160	No 4 fold rise in titres
6	887599 -1 -2	2 10	1:80	1:160	No 4 fold rise in titres
7	769201 -1 -2	11 21	1:80	1:80	No 4 fold rise in titres
8	664444 -1 -2	4 14	1:80	1:160	No 4 fold rise in titres
9	773505 -1 -2	3 10	1:80	1:80	No 4 fold rise in titres
10	846322 -1 -2	2 9	1:160	1:160	No 4 fold rise in titres
11	770095 -1 -2	4 13	1:40	1:40	No 4 fold rise in titres
12	831983 -1 -2	3 13	1:160	1:80	No 4 fold rise in titres
13	767932 -1 -2	3 11	1:40	1:160	**4 fold rise in titres**
14	908104 -1 -2	3 10	1:40	1:160	**4 fold rise in titres**
15	770686 -1 -2	2 10	1:80	1:320	**4 fold rise in titres**

Chart 14: Results of Acute-convalescent paired samples in IIFA test (n=15)

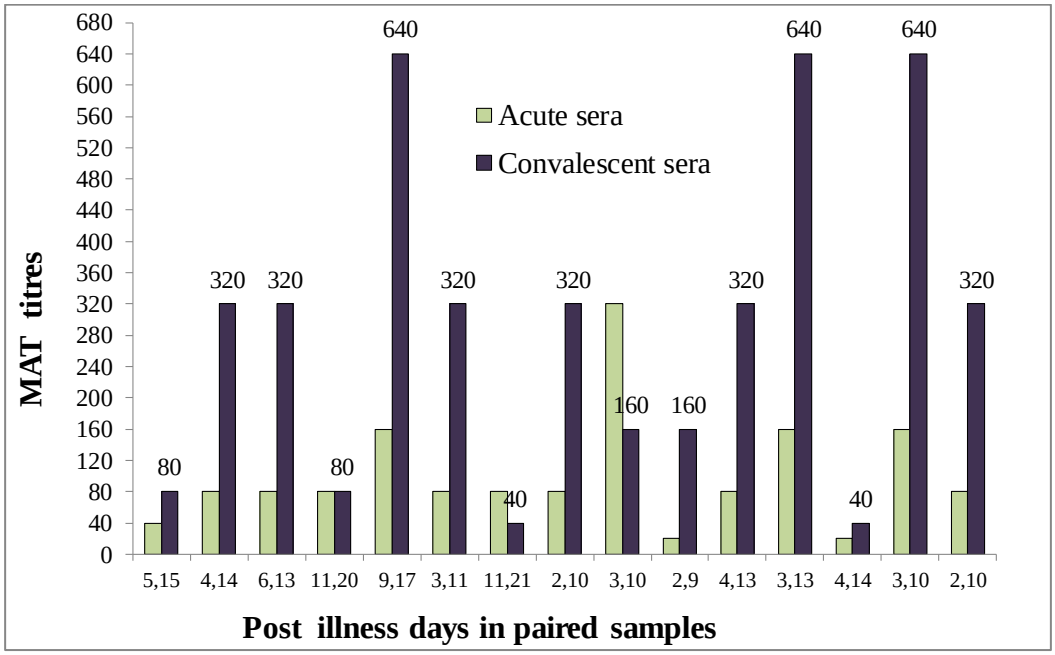


IIFA showed 4 fold rise in titres only in 2 (13.33%) of the 15 paired samples tested.

Table 24: Results of MAT on Acute – Convalescent Paired samples (n=15)

Sl no.	Specimen no.	Days PI	MAT Titres		Interpretation
			Acute	Convalescent	
1	887599 -1 -2	2 10	1:**80**	1:**320**	**4 fold rise in titres**
2	767733 -1 -2	4 14	1:80	1:320	**4 fold rise in titres**
3	767810 -1 -2	6 13	1:**80**	1:**320**	**4 fold rise in titres**
4	908104 -1 -2	3 10	1:**160**	1:**640**	**4 fold rise in titres**
5	767944 -1 -2	9 17	1:**160**	1:**640**	**4 fold rise in titres**
6	767932 -1 -2	3 11	1:80	1:320	**4 fold rise in titres**
7	831983 -1 -2	3 13	1:**160**	1:**640**	**4 fold rise in titres**
8	770686 -1 -2	2 10	1:**80**	1:**320**	**4 fold rise in titres**
9	846322 -1 -2	2 9	1:**20**	1:**160**	**4 fold rise in titres**
10	770095 -1 -2	4 13	1:**80**	1:**320**	**4 fold rise in titres**
11	767200 -1 -2	5 15	1:40	1:80	No 4 fold rise in titres
12	773505 -1 -2	3 10	1:**320**	1:**160**	No 4 fold rise in titres
13	664444 -1 -2	4 14	1:**20**	1:**40**	No 4 fold rise in titres
14	769201 -1 -2	11 21	1:**80**	1:**40**	No 4 fold rise in titres
15	767839 -1 -2	11 20	1:80	1:80	No 4 fold rise in titres

Chart 15: Results of Acute-convalescent paired samples in MAT and IIFA (n=15)



MAT showed 4 fold rise in titres in 10 (66.66%) of the 15 paired samples tested.

6. DISCUSSION

Leptospirosis is an endemic zoonotic disease in many parts of India. Periodic epidemics or outbreaks of leptospirosis have been reported from many states in Southern India when favourable conditions exist. [4, 48, 59] Leptospirosis has also been reported from many parts of Karnataka. [4, 5, 6] By our hospital records and laboratory statistics of leptospirosis cases by IgM ELISA, in the last 3 years, it is seen that leptospirosis is a significant public health disease in the local population. Earlier reports of leptospirosis are available in kolar region.

Over a period of 1 year and 7 months from January 2012 to July 2013, among 2362 patients who were screened by serological testing in whom leptospirosis was suspected, majority of the patients belonged to 20-29 years age group accounting for 28.7% of the patients. This was followed by 16.51% patients in 30-39 year age group. Thus, most of the patients in our study, with the clinical suspicion of leptospirosis were in the 3rd and 4th decade of life. This could be explained, as this subset of population indulges more in outdoor working activity, they are more prone to develop such infections. We found a slight preponderance of male patients over female patients. Males accounted for 55.38% of the patients where as female patients accounted for 44.62% of the patients screened.

The month wise distribution shows that there is a variation in the distribution of cases suspected of leptospirosis. Majority of the cases accounting for 45.21% of suspected leptospirosis presented during the months of January and February 2012.

Over a period of 1year and 7 months, the seropositivity rate among 2362 patients who presented to our hospital with fever and in whom leptospirosis was suspected, was found to be 3.3% by IgM ELISA. Other patients would have had fever

from other causes which are transmitted in the local community like Dengue, Malaria, Typhoid and other infections. This rate is less when compared to the seropositivity rate in our hospital in the previous years; 21.8%, 11.2% and 8% in 2011, 2010 and 2009 respectively. Thus, there is wide fluctuation in the seropositivity rate among patients with suspected leptospirosis visiting our hospital in different years. Various studies in India have shown almost similar seropositivity rates. According to the ICMR survey, seropositivity rate is highest n southern India with 25.6%. [48] A study from Mumbai in 1967, conducted on patients with acute febrile illness showed a seropositivity rate of 30%. In 1983 in Madras, a study on cases of febrile illness showed 18% seropositivity. [59] In 1995, a study from Pondicherry documented a seropositivity rate of 12%. [59] In Bangalore in 1988, a serological survey reported a seropositivity rate of 6%. [5]

Among these 78 patients who had a serological evidence of leptospirosis by IgM ELISA test, during the study period, adults in the age group 20-40 years constituted for the majority of cases accounting for 51.28% of the patients. We think that this predominance could be due to the exposure of these people during occupational activities to the water and soil sources, contaminated with rodent urine. Our findings are similar to the findings in other studies. A study in Chennai, reported that the disease occurred in 44.25% of the patients who were in 20-40 years age group. [65] Other studies from Gujarat and Pondicherry have reported that majority of the patients were in the 20-40 years age group. [82, 83]

Women accounted for 65% while men accounted for 35% of the 78 patients who were positive by IgM ELISA test. This could be due to more exposure of women than men in peri domestic area, where rodents are more prevalent and also may be due to non agrarian activity of men during certain periods in a year. This finding is in

contrast with other studies from Chennai, Gujarat, Pondicherry and Mumbai, where there is male preponderance, as males are more involved in outdoor activities making them vulnerable to acquire this infection. [65, 82, 83, 84]

In 78 IgM ELISA positive cases, the month wise distribution showed time clustering of cases in January and February 2012 which accounted for 55% of the cases. Such a peak occurring during one or two months of 2012 and cases continuing to occur throughout the year are features of endemo-epidemic pattern of leptospirosis. No such peaks or increase in the number of cases were seen in the months of January and February of 2013. This may be related to the transmission of disease in the community. As most of our patients come from rural agrarian community, lack of optimal rainfall leads to decreased agricultural activities during some part of the years. During non agrarian or non cultivation periods, the incidence of the infection is also less due to lower exposure. This may explain the rural pattern of Leptospirosis due to variation in agrarian activity. [85] Most of the studies from India have reported that the infection rate is higher during monsoon and post monsoon periods from July to November months. [56, 57, 65, 82] An association between heavy rainfall and human leptospirosis has been reported in India from Chennai, Orissa and Mumbai in the years 1990-91, 1999 and 2000 respectively. However, leptospirosis continues to occur in the above places throughout the year almost to the same extent. This may be true with the urban pattern of leptopirosis. [30, 85] But in the draught prone rural areas like Kolar region, this urban pattern may not be seen.

Two hundred and fifty patients with fever during the above period were screened for leptospirosis by both IgM ELISA and IIFA and positives detected by them were subjected to MAT for confirmation.

Different serological tests detect different classes of antibodies. IgM ELISA test detects IgM class of antibodies. IIFA test done using anti human IgG FITC conjugate detects IgG class of antibodies. Both IgM and IgG classes of antibodies participate in MAT, but later in the course of the disease.

ELISA is an easily performed, commercially available, rapid screening test. We wanted to compare the results of IgM ELISA with IIFA test using anti IgG FITC conjugate which could be easily procured by us. To evaluate the positives detected by these tests, we performed MAT. Many studies report that ELISA has a good sensitivity and specificity. [8, 83, 86] Few studies report that ELISA has a less sensitivity and specificity. [10, 69] ELISA has got the chances of giving positive results in cases other than leptospirosis like Dengue, Malaria, Typhoid and Hantavirus infections due to immunological cross reactivity. This has been reported to account for the significant false positivity associated with IgM ELISA. [8, 9, 26] The test also is reported to give false negativity. The reasons for false negativity are thought to be related to variation in the population in time when IgM antibodies develop. [9] This may be due to the nature of immune response where there is variation in the appearance of IgM and IgG antibodies.

By taking in to consideration, both the geometric mean titre of IIFA estimated in our study and as recommended by Torten et al and others, we have taken 1:80 as the cut off titre in IIFA test. [67, 69, 70] At 1:80 as a cut off titre, among 50 samples which were positive by IgM ELISA test, only 68% were positive by IIFA where as another 32% were tested negative by IIFA. Among 200 samples which were negative by IgM ELISA test, 2% were positive by IIFA. The overall concordance (percentage of agreement) between IgM ELISA and IIFA tests is found to be 92%. But in actual, as per the convention of adding 3 standard deviation of geometric mean titre estimated in

our study, 1:40 should have been taken as a cut off titre. We took 1:80 as cut off, to increase the specificity and also as in serology there is no significant difference between two fold dilutions. If a titre of 1:40 is taken as a cut off titre, the IIFA positivity among ELISA positive cases increases from 68% to 88%. The other significant finding which can be seen at 1:40 cut off titre is that, 12.5% cases which are negative for IgM class of antibodies in ELISA are positive in IIFA test. One of the reasons for this difference in results can be thought of, to be due to use of anti human IgG and not anti human IgM FITC conjugate in IIFA test. In this light, further evaluation has to be done using MAT. It is important to note here, that antihuman IgG which we have used in our study is easily available and cheaper; each test costing about Rs. 30/-. Whereas, antihuman IgM is not easily available in India, had to be imported and is expensive.

IIFA developed by Torten and colleagues was found to be a good alternative test for the much laborious and tedious MAT test. [67, 69,] IIFA has an advantage that the slides with fixed antigen can be stored in a closed slide box at +4ºc for up to 3 months without any deterioration of the antigen. [67]

Most of the studies to evaluate IIFA have been done in Thailand. There are very few studies from India. A study from Thailand in the year 2000, evaluating five different serological tests for the diagnosis of leptospirosis have reported a sensitivity of 92.5% and a specificity of 95% for IIFA. [69] Another study from Thailand in the year 2004, have shown similar findings reporting a sensitivity of 91.9% and a specificity of 100% for IIFA. [10] However, they have used anti human IgM FITC conjugate. A study from India evaluating IgM specific IIFA in diagnosing an outbreak has reported good sensitivity for IIFA. But the sample size evaluated was very less. [68]

In contrast to the above studies, a study from Thailand in 1995 reports a sensitivity of only 48% in IIFA with reference to MAT. However, the specificity was good at 97%. [70] Other studies from Iran and Uruguay report that IIFA is less sensitive and specific in diagnosing leptospirosis. Also, they opine that the readings are observer dependent based on personal skills and experience, making it difficult to be used in routine practice. [87, 88] Our results are in line with these observations. However, the lower titres of 1:40 may be a feature of immune response early in the course of infection. We suggest that the patients with such titres should be evaluated further by MAT and also by testing paired samples.

We performed MAT on the samples which were positive either by IgM ELISA or IIFA test to confirm the diagnosis, as MAT is the reference standard test. As MAT is considered as diagnostic, only when fourfold rise in titres is demonstrated among paired samples, which practically is difficult to achieve at, one has to consider fixing a cut off titre in MAT for a single sample available to make a diagnosis. This depends on the baseline titres of MAT in the community in a particular geographical region which requires studies. Several researchers usually suggest a titre of 1:80 or 1:100 as a significant titre for diagnosis without considering the endemicity or baseline titres in the community. [26] The cut off titre in MAT was fixed at 1:80 according to these recommendations and also as in accordance with other studies. [9, 83, 84, 86] Overall, in 54 cases, MAT was positive in 81.48% and negative in 18.52% of the samples tested. Among 50 samples positive by IgM ELISA, 86% were positive by MAT and 14% were negative by MAT. These 14% of the cases may not be the cases of leptospirosis or it is also possible that the agglutinin titres which are detected by MAT would not have developed in the patients' sera still, especially if the duration of illness is less than 8 days. We could obtain paired samples in three of the patients who were

positive by IgM ELISA and negative by MAT. One of these patients showed fourfold rise in titres. This accounts for 88% positivity by MAT among IgM ELISA positive cases.

The congruity between all the three tests is found to be 55.56%. Concordance between IgM ELISA test and IIFA test is 92%. Evaluating the positive result in any two of the three serological tests, for a case to be considered as a presumptive case of leptospirosis, 88.88% of the 54 cases tested can be considered as presumptive cases of leptospirosis.

Paired sera were obtained from a total of 15 patients who were positive by IgM ELISA test. They were subjected to both IIFA test and MAT. IIFA test showed fourfold rise in titres only in 2 (13.33%) cases. Hence, IIFA is not a good test to demonstrate fourfold rise in titres of the antibodies which is diagnostic. This is in contrast with the other study where IIFA was found to be a good test to demonstrate four-fold rise in antibody titres. [70] MAT could demonstrate four-fold rise in titres of the antibody in 10 (66.66%) of the 15 patients. Other 5 paired samples showed persistent high titres, even though they did not show the four-fold rise in titres. Among 7 (14%) cases which were positive by IgM ELISA test and negative by MAT, paired samples were available in 3 patients and only one of them showed fourfold rise in antibody titres.

The clinical details were obtained for 50 cases that were positive by IgM ELISA test. All the patients had fever. In our study, 96% met Modified Faine's criteria and most of them had myalgia, headache, vomiting and conjunctival suffusion. Only three patients had jaundice and two of them had elevated liver enzymes supporting the common finding that Hepato-renal syndrome is a misnomer

for leptospirosis. We also had respiratory infection in 20% of the patients as reported by other studies in Andaman Islands. [50] Treatment was instituted immediately after IgM ELISA results. IV Crystalline Penicillin/ Ceftriaxone therapy has been proved to be useful in our patients with leptospirosis described here except in two patients. Despite effective therapy, one patient with Acute respiratory distress syndrome (ARDS) and the other with multiple organ dysfunction syndrome (MODS) expired.

7. SUMMARY

Our study included the screening of serum samples of patients with fever and clinically suspected of Leptospirosis, visiting our hospital during a period of 1 year and 7 months from January 2012 to July 2013 by IgM ELISA. We evaluated IgM ELISA and IIFA tests as screening tests for the diagnosis of leptospirosis and compared the positives detected by these tests by MAT which is a confirmatory test.

Serum samples from 2362 patients with fever and clinical suspicion of leptospirosis during the acute phase of illness were screened by IgM ELISA. Seropositivity rate is found to be 3.3%. Majority of the IgM ELISA positive cases were adults belonging to 3rd and 4th decades of life accounting for 51.28% of the patients. Preponderance of women is seen in our study who accounted for 65% of the cases. Most of the cases were seen during January and February 2012 accounting for 55% of the cases. Thus, leptospirosis seems to be an endemic disease in Kolar region with epidemic peaks in some of the years.

Fifty samples which were positive for IgM class of antibodies and 200 samples which were negative for IgM class of antibodies by ELISA were further tested by IIFA to detect concordance between these two tests. IIFA could detect only 68% of the cases that were positive by ELISA and it detected 2% of the cases that were negative by ELISA. However, the concordance between the two tests was found to be 92%. Concordance was calculated by taking both the positive and negative results of ELISA and IIFA.

All the positives by either IgM ELISA or IIFA in the subpopulation of patients were confirmed by using MAT. MAT confirmed 88% of the cases that were positive

by IgM ELISA, thereby accounting for a false positivity rate of 12% in IgM ELISA. Whereas, MAT confirmed only 81.58% of the cases that were positive by IIFA.

The congruity between all the three tests is found to be 55.56%. Evaluating the positive result in any two of the three serological tests, for a case to be considered serologically as a presumptive case of leptospirosis, 88.88% of cases fitted into a serologically presumptive diagnosis of leptospirosis.

Paired sera obtained from a total of 15 patients who were positive by IgM ELISA test was subjected to both IIFA test and MAT parallelly. MAT demonstrated fourfold rise in titres in 66.66% of the cases which is serologically diagnostic of Leptospirosis, whereas IIFA did so only in 13.33% of the cases. Therefore, MAT was found to be an ideal test to demonstrate fourfold rise in titres which is diagnostic of leptospirosis where as IIFA was not found to be a good test to demonstrate fourfold rise in titres.

8. CONCLUSION

In the study presented in dissertation, screening of 2362 acute sera of the patients suspected of Leptospirosis by IgM ELISA over a period of 1 year and 7 months showed a seropositivity rate of 3.3%.

Among the IgM positive cases, adults in the age group 20- 40 years constituted for the majority of cases accounting for 51.28% of the patients. Preponderance of women was seen in our study who accounted for 65% of the cases.

There was time clustering of cases of Leptospirosis during the months of January and February 2012, accounting for 55% of the cases.

A subset of 50 sera which were positive by IgM ELISA and 200 sera which were negative by IgM ELISA were subjected to IIFA. Positives detected by either of these two tests were confirmed by MAT.

Congruity between all the three serological tests was found to be 55.56%. Concordance between IgM ELISA and IIFA was found to be 92%.

IIFA could not diagnose 32% of the cases which were positive by IgM ELISA. However, considering MAT as a reference standard test, IIFA missed only 13.48% of the cases.

MAT was positive in 88% of the cases which were positive by IgM ELISA. This accounts for 12% false positivity by ELISA. MAT was positive in 81.58% of the cases which were positive by IIFA.

Overall 88.88% of the cases gave a presumptive diagnosis of Leptospirosis, considering a presumptive case as the one which is positive in any two of the three serological tests done.

Among 15 paired (Acute-convalescent) samples, MAT demonstrated fourfold rise in titres in 66.66% of the cases which is serologically diagnostic of Leptospirosis, whereas IIFA did so only in 13.33% of the cases.

Our study showed that IgM ELISA can be considered as a good screening test. IIFA did not show any additional advantage for the diagnosis of leptospirosis. However, further study on IIFA using anti human IgM FITC conjugate is necessary for proper evaluation. MAT is an ideal test to demonstrate four-fold rise in antibody titres in paired samples and IIFA does not seem to be an ideal test for paired samples.

9. BIBLIOGRAPHY

1. Faine S, Adler B, Bolin C, Perolat P. Leptospira and Leptospirosis. 2nd edn, Melbourne, Medisci 1999.
2. Levett PN. Leptospirosis. Clin Microbiol Rev; 2001: 296 – 326.
3. Adler B, Faine S. Leptospira. In: Parker MT, Collier LH, eds. Topley and Wilson's Principles of Bacteriology, Virology and Immunology. Vol 2, 8th edn. London: Edward Arnold, 1990:1865-1884.
4. Shivakumar S. Leptospirosis-Current scenario in India. Medicine update 2008; 18: 799-809.
5. Madhusudhana SN, Bharghawa SK. Serological survey of Leptospirosis in and around Bangalore. Indian J pathol Microbiol 1988; 32: 60-63.
6. Masali KA, Pulare MV, Kachare VJ, Patil MB, Reddi S. Control and Prevention of Rat Fever (Leptospirosis) Outbreak in Six Villages of Raichur District, Karnataka. J Indian Med Assoc 2007; 105: 633-636.
7. Human Leptospirosis: Guidance for Diagnosis, Surveillance and Control. WHO Library Cataloguing-in-publication Data. World Health Organization. Malta: 2003.
8. Bajani MD, Ashford DA, Bragg SL, Woods CW, Aye T, Spiegel RA et al. Evaluation of four commercially available rapid serologic tests for diagnosis of Leptospirosis. J Clin Microbiol 2003; 41: 803-809.
9. Swapna RN, Tuteja U, Nair L, Sudarsana J. Seroprevalence of Leptospirosis in high risk groups in Calicut, North Kerala, India. Indian J Med Microbiol 2006; 24: 349-352.

10. Kemapunmanus M, Sretrirutchai S, Khuntikij P, Pradutkanchana S, Pradutkanchana J. A prospective evaluation of four Immunodiagnostic assays for Human Leptospirosis. Southeast Asian J Trop Med public Health 2004; 35: 863-867.
11. Guptha KRL. Madhava nidhana, Ayurvedic system of pathology. Indian Medical Science Series 1997; 7: 39-43.
12. Marr JS, Cathey JT. New Hypothesis for Cause of Epidemic among Native Americans, New England, 1616–1619. Emerg Infect Dis 2010; 16: 281-286.
13. Abdollahpour G. A review on Leptospirosis. 2013: Available from www.leptolab.ut.ac.ir.
14. Bibliography @ Who Named It. Com.
15. Stimson AM: Note on an organism found in yellow-fever tissue. Public health reports Washington 1907; 22: 541.
16. Inada R, Ido Y, Hoki R, Kaneko R, Ito H. The etiology, mode of infection, and specific therapy of weil's disease (spirochaetosis icterohaemorrhagica). J Exp Med 1916; 23: 377-402.
17. Guidelines for the Prevention of Leptospirosis. Communicable Disease Control and prevention 2008:1-11.
18. Zinsser H. Rats, Lice and History. Back Bay Books 1965: 199.
19. W.E. Williams. Browning, The Pied Piper Of Hamelin 1977: 97 -101.
20. Vijayachari P, Sugunan AP, Shriram AN. Leptospirosis: an emerging global public health problem. J Biosci 2008; 4:557-569.
21. Sritharan M. Insights into Leptospirosis, a Neglected Disease. In Dr. Jacob Lorenzo- Morales ed. Zoonosis, ISBN 2012: 167-192.

22. Abdussalam M, Alexander AD, Babudieri B, Borg-Peterson C, Eichhorn EA, Galton MM et al. Classification of leptospires and recent advances in leptospirosis. Bull Wrld Hlth Org 1965; 32: 881 – 891.
23. Adler B, Murphy AM, Locarnini SA, Faine S. Detection of specific anti-leptospiral immunoglobulins M and G in human serum by solid-phase enzyme-linked immunoabsorbent assay. J Clin Microbiol1980; 11: 452–457.
24. Wang Z, Jin L, Wegrzyn A. Leptospirosis vaccines. Microbial Cell Factories 2007; 6: 1-10.
25. Koizumi N, Watanabe H. Leptospirosis vaccines: Past, present, and future. Symposium 2005; 51: 210-214.
26. Leptospirosis Laboratory Manual. Regional Medical Research Centre, Indian Council of Medical Research, Port Blair. World Health Organization: 2007.
27. Muthusethupathi MA, Shivakumar S, Suguna R, Jayakumar M, Vijaykumar R, Everard COR et al. Leptospirosis in Madras: a clinical and serological study. J Assoc Phys India 1995; 43: 456-458.
28. Prabhakar PK, Harish BN, Rao RS. Seroprevalence of leptospirosis among febrile and jaundice patients. Indian J Med Microbiol 1995; 13: 189-191.
29. Ratnam S, Subramanian S, Madanagopalan N, Sundararaj T, Jayanthi V. Isolation of leptospires and demonstration of antibodies in human leptospirosis in Madras, India. Trans Roy Soc Trop Med Hyg 1983; 77: 455 - 458.
30. Prasad SR, Rajini M. Leptospirosis; An Overview. The J Acad Clin Microbiol 2008; 10: 89-97.
31. Adler B, Moctezuma ADLP. Leptospira and leptospirosis. Veterinary Microbiology 2010; 140: 287-296.

32. Coleman TJ. Leptospira. In: Greenwood D, Slack RCB, Peutherer JF, eds. Medical Microbiology. 16th edn. London: Churchill Livingstone, Elsevier 2006: 352- 357.

33. Haake DA, Matsunaga J. Characterization of the leptospiral outer membrane and description of three novel leptospiral membrane proteins. Infect Immun 2002; 70: 4936-4945.

34. MicrobialGenetics. In: Brooks GF, Carroll KC, Butel JS, Morse SA, Meitzner TA, eds. Jawetz, Melnick, & Adelberg's Medical Microbiology. 25thedn. New York: McGraw Hill, Lange Medical 2010: 98-99.

35. David HSU, Pan MJ, Zee YC, Lefebvre RB. Unique ribosome structure of Leptospira interrogans is composed of four rRNA components. J Bacteriol 1990; 172: 3478.

36. Victoria B, Ahmed A, Zuerner RL, Ahmed N, Bulach DM, Quinteiro J et al. Conservation of the S10-spc-a Locus within Otherwise Highly Plastic Genomes Provides Phylogenetic Insight into the Genus Leptospira. PLoS ONE 2008; 3: e 2752.

37. Bourhy P, Salau NL, Lajus AL, Me digue C, Eude CB, Picardeau M. A Genomic Island of the Pathogen Leptospira interrogans Serovar Lai Can Excise from Its Chromosome. J Infect and Immun 2007; 677–683.

38. Ricaldi JN, Fouts DE, Selengut JD, Harkins DM, Patra KP, Moreno A et al. Whole Genome Analysis of Leptospira licerasiae Provides Insight into Leptospiral Evolution and Pathogenicity. PLOS Neg trop dis 2012; 6: e 1853.

39. Ellinghausen HC Jr. Growth temperatures, virulence, survival, and nutrition of leptospires. J Med Microbiol 1973; 6: 487- 497.

40. Bey RF, Johnson RC. Protein-free and low-protein media for the cultivation of Leptospira. Infect Immun 1978; 19: 562-569.
41. Griffith ME, Horvath LL, Mika WV, Hawley JS, Moon JE, Hospenthal DR et al. Viability of Leptospira in BacT/ALERT MB media. Diagn Microbiol Infect Dis 2006; 54: 263-266.
42. Cinco M, Banfi E, Soranzo MR. Studies on the interaction between macrophages and leptospires. J Gen Microbiol 1981; 124: 409-413.
43. Levett PN, Haake DA. Leptospira species (Leptospirosis) In: Mandell Douglas, Bennett's. eds. Principles and practice of Infectious Diseases. 7th edn Philadelphia: Elsevier 2010; 2: 3059-3065.
44. Dassanayake DL, Wimalaratna H, Nandadewa D, Nugaliyadda A, Ratnatunga CN, Agampodi SB. Predictors of the development of myocarditis or acute renal failure in patients with leptospirosis: an observational study. BMC Infect Dis 2012; 12: 4.
45. Rathinam SR. Ocular manifestation of leptospirosis. J Postgrad Med 2005; 51: 189-194.
46. Rathinam SR, Rathnam S, Selvaraj S, Dean D, Nozik RA, Namperumalsamy P. Uveitis associated with an epidemic outbreak of leptospirosis. Am J Ophtalmol 1997; 124: 71-79.
47. Turgut M, Sunbul M, Bayirli D, Bilge A, Leblebicioglu H, Haznedaroglu I. Thrombocytopenia complicating the clinical course of leptospiral infection. J Int Med Res 2002; 30: 535-540.
48. Dutta TK, Christopher M. Leptospirosis – An Overview. J Assoc Phys India 2005; 52: 545-551.

49. Terpestra WJ. Historical perspectives in leptospirosis. Indian J Med Microbiol 2006; 24 (suppl): 316-320.
50. Vijayachari P, Sugunan AP, Sharma S, Roy S, Nataraja Sreenivasan K, Sehgal SC. Leptospirosis in Andaman Islands, India. Trans Roy Soc Trop Med Hygiene 2008; 162: 117-122.
51. Shivakumar S. Leptospirosis –Evaluation of clinical criteria. J Assoc Phys India 2003; 51: 329-330.
52. Shivakumar S, Shareek PS. Diagnosis of Leptospirosis –Utilizing modified faine's criteria. J Assoc Phys India 2004; 52: 678-679.
53. Mohan Rao AMK. Preventive measures for leptospirosis: rodent control. Indian J Med Microbiol 2006; 24 (suppl): 325-328.
54. Kouadio IK, Aljunid S, Kamigaki T, Hammad K, Oshitani H. Expert reviews. Infectious diseases following natural disasters: prevention and control measures. Expert Rev. Anti Infect Ther 2012; 10: 95–104.
55. De A, Varaiya A, Pujari A, Mathur M, Bhat M, Karande S etal. An outbreak of Leptospirosis in Mumbai. Indian J Med Microbiol 2002; 3: 153-155.
56. Sehgal SC, Sugunan AP, Vijayalakshmi P. Outbreak of leptospirosis after the cyclone in Orissa. Nat Med J India 2002; 15: 22-23.
57. Karande S, Kulkarni H, Kulkarni M, De A, Varaiya A. Leptospirosis in children in Mumbai slums. Indian J of Pediatrics 2002; 69: 855-858.
58. Demers RY, Thiermann A, Demers P, Frank R. Exposure to Leptospira icterohaemorrhagiae in inner-city and suburban children: a serologic comparison. J Fam Pract 1983; 17: 1007-1011.
59. Sambasiva RR, Naveen G. Bhalla P and Agarwal SK. Leptospirosis in India and the Rest of the World. Braz J Infect Dis 2003; 7: 178-193.

60. Leggat D. "Rowing: Rare disease kills rowing great". The newzealand herald. Oct 27, 2010.

61. Seamark M. Rat disease kills Redgrave's gold medal partner: Rower dead in days from water-borne illness. Mail online, 26 October 2010. www.dailymail.co.uk.

62. Sharma M, Yadav A. Leptospirosis: Epidemiology, Diagnosis, and Control. J Infect Dis Antimicrob Agents 2008; 25: 93-103.

63. Ratnam S. Leptospirosis: an Indian perspective. Indian J Med Microbiol 1994; 12: 228–239.

64. Everard, JD, Everard COR. Leptospirosis in the Caribbean. Rev Med Microbiol 1993; 4: 114–122.

65. Arumugam G, Jacob SM, Anitha D, Rajappa SM. Occurrence of leptospirosis among suspected cases in Chennai, Tamil Nadu. Indian J Pathol Microbiol 2011; 54: 100-102.

66. Sharma KK, Kalawat U. Early diagnosis of leptospirosis by conventional methods: one-year prospective study. Indian J Pathol Microbiol 2008; 51: 209-211.

67. Torten M, Shenberg E, Van Der Hoeden J. The use of Immunofluorescence in the diagnosis of human leptospirosis by a genus specific antigen. J Infect Dis 1966; 116: 537-543.

68. Joshi S, Bal A, Bharadwaj R, Kumbhar R, Kagal A, Arjunwadkar V. Role of I_gM specific Indirect immunofluorescence assay in diagnosing an outbreak of Leptospirosis. Indian J Pathol Microbiol 2002; 45: 75-78.

69. Naigowit P, Wangroongsarb P, Petkanchanapong W, Luepaktra O, Warachit P. A Comparative Evaluation of Different Methods for the serological Diagnosis of Leptospirosis. J Trop Med Parasitol 2000; 23 : 59-65.

70. Appassakij H, Silpapojakul K, Wansit R, Woodtayakorn J. Evaluation of the Immunofluorescent antibody test for the diagnosis of Human Leptospirosis. Am J Trop Med Hyg 1995; 52: 340-343.

71. Sehgal SC, Vijayachari P, Subramaniam V. Evaluation of Leptospira Micro Capsular Agglutination Test (MCAT) for sero-diagnosis of leptospirosis. Indian J Med Res 1997; 106: 504-507.

72. Sehgal SC, Vijayachari P, Sharma S, Sugunan AP. LeptoDipstick - a rapid and simple method for serodiagnosis of leptospirosis in acute stage. Trans Roy Soc Trop Med Hyg 1999; 93:161-164.

73. Vijayachari P, Sehgal SC. Recent advances in the laboratory diagnosis of leptospirosis and characterization of leptospires. Indian J Med Microbiol 2006; 24 (Suppl): 320-322.

74. Cole JR, Sulzer CR, Pursell AR. Improved microtechniques for the leptospiral agglutination test. Applied Microbiology 1973; 25: 976-980.

75. Vijayachari P, Sugunan AP, Sehgal SC. Role of Microscopic Agglutination Test (MAT) as a diagnostic tool during acute stage of leptospirosis in low and high endemic areas. Indian J Med Res 2001; 114: 99-106.

76. Kuriokose M, Paul R, Joseph MR, Sugathan S, Sudha TN. Leptospirosis in a midland rural area of kerala state. Indian J Med Res 2008; 128: 307-312.

77. Gouse M, Maulana AB, Mohamed Ali MG, Sarasa VD. A two-year study of the efficacy of Azithromycin in the treatment of leptospirosis in humans. Indian J Med Microbiol 2006; 24 (suppl): 345-346.

78. Baker FJ, Breach MR. Medical microbiological techniques. London: Butterworths, 1980: 24.
79. Indirect Immunofluorescence test (IIFT). Available from http://tesla.rcub.bg.ac.rs/lepto/lab.
80. Philip FJ, John WJ. How not to lie with statistics: the correct way to summarize bench-mark results. Communications of the ACM 1986; 29: 218-221.
81. Langenbucher J, Labouvie E, Morgenstern J. Methodological developments. Measuring Diagnostic Agreement. J Consult Clin Psychology 1996; 64: 1285-1289.
82. Patel BK, Gandhi SJ, Desai DC. Clinico-epidemiological aspects of leptospirosis in south Gujarat. Indian J Med Microbiol 2006; 24 (suppl): 322-326.
83. Shekatkar S, Harish B, Menezes G, Parija S. Clinical and serological evaluation of Leptospirosis in Puducherry, India. J Infect Dev Ctries 2010; 4: 139-143.
84. Mathur M, De A, Turbadkar D. Leptospirosis outbreak in 2005: L.T.M.G Hospital experience. Indian J Med Microbiol 2009; 27: 153-155.
85. Sehgal SC. Epidemiological patterns of leptospirosis. Indian J Med Microbiol 2006; 24: 310-311.
86. Cumberland P, Everard COR, Levett PN. Assessment of the efficacy of an I_gM ELISA and Microscopic agglutination test (MAT) in the diagnosis of Acute Leptospirosis. Am J Trop Med Hyg 1999; 61: 731-734.
87. Dezfully NK, Mehrabian S. Evaluation of two Immunodiagnostic assays (MAT and IFA) for Human Leptospirosis in Gilan province – Iran. Jundishapur J Microbiol 2012; 5: 582-584.

88. Schelotto F, Hernandez E, Gonzalez S, Delmonk A, Ifran S, Flores K etal. A Ten-year follow-up of Human Leptospirosis in Uruguay: An unresolved Health problem. Rev Inst Med Trop Sao Paulo 2012; 54: 69-75.
89. Murhekar MV, Sugunan AP, Vijayachari P, Sharma S,Sehgal SC. Risk factors in the transmission of leptospiral infection. Indian J Med Res 1998; 107: 218-223.