CORRELATION OF DIFFERENT LABORATORY PARAMETERS IN THE DIAGNOSIS OF LEPTOSPIROSIS

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CERTIFICATE

This is to certify that this dissertation titled **"CORRELATION OF DIFFERENT LABORATORY PARAMETERS IN THE DIAGNOSIS OF LEPTOSPIROSIS"** is a bonafide record of work done by **Dr. B. ANANTHI,** during the period of her Post graduate study from June 2006 to March 2009 under guidance and supervision in the Institute of Microbiology, Madras Medical College and Government General Hospital, Chennai-600003 in partial fulfillment of the requirement for **M.D. Microbiology** Degree Examination of The Tamilnadu Dr. M.G.R. Medical University to be held in March 2009.

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DECLARATION

I declare that the dissertation entitled ***"CORRELATION OF DIFFERENT LABORATORY PARAMETERS IN THE DIAGNOSIS OF LEPTOSPIROSIS"*** *submitted by me for the degree of M.D. is the record work carried out by me during the period of* ***March 2007 – February 2008*** *under the guidance of* ***Dr.G.SUMATHI, M.D.,Ph.D.,*** *Director and Professor, Institute of Microbiology, Madras Medical College, Chennai. This dissertation is submitted to the Tamilnadu Dr.M.G.R. Medical University, Chennai, in partial fulfillment of the University regulations for the award of degree of M.D., Branch IV (Microbiology) examination to be held in March 2009.*

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Introduction

INTRODUCTION

India with long coastline, has one of the major important coastal agro ecosystem that supports livelihood of several million people. However this system is highly fragile. Due to the rapid ecological changes during the past decade many zoonotic diseases have emerged and resulted in epidemics leading to significant morbidity and mortality in humans. Leptospirosis is one among them.[29]

Leptospirosis is a zoonotic infection caused by the spirochaete of the Genus Leptospira. It affects humans worldwide, in both urban and rural areas and in temperate and tropical climates.[46] Pathogenic leptospires live in the kidney of natural hosts, predominantly mammals and are excreted with the urine into the environment where they survive for several months in humid, warm and slightly alkaline conditions. Humans are accidental hosts and are infected by contact with an environment contaminated by urine of shedder hosts such as rodents, cattle and dogs.[90]

The route of infection is through abraded skin and mucus membranes. Pathogenic leptospires rapidly invade the bloodstream and causes disruption of the integrity of the cell membrane of the endothelial cells lining the blood vessels in all parts of the body. Capillary leakage and hemorrhages occur in all the organs and tissues, particularly the lungs, omentum and pericardium. Ischaemia from damage to blood vessels in the renal cortex leads to renal tubular necrosis, particularly of the proximal convoluted tubules. This may lead to renal failure that can be fatal. Liver cell necrosis caused by ischaemia and the destruction of hepatic architecture leads to the characteristic jaundice of the severe type of leptospirosis. Blood clotting mechanisms are affected by liver failure, aggravating the haemorrhagic tendencies.[91] Thus the clinical spectrum of leptospirosis is very wide with mild anicteric

presentation at one end to severe leptospirosis with severe jaundice and multiple organ involvement on the other end.[29]

The clinical presentation of leptospirosis is a **biphasic** illness. The first phase of the disease is commonly referred to as the septicemic phase. It is characterised by fever, headache, myalgia, conjunctival congestion and a host of non-specific features that may include mild cough, lymphadenopathy, rash, anorexia, nausea, and vomiting. This phase is followed by a brief afebrile period of variable duration that, in turn, is followed by the immune phase of illness. The common organs involved during immune phase are the liver and kidneys. Both organ derangements are reversible. The severe form of leptospirosis, commonly known as “Weil’s disease”, is characterised by a fulminant course with rapid onset of hepatic and renal failure and high mortality.[3]

Eventhough it is a potentially serious disease it can be treated. Early diagnosis and appropriate treatment will halt the progression of the disease and reduces the mortality and morbidity of this zoonotic infection.

Its symptom mimicks many other diseases like dengue, viral hepatitis, meningitis, influenza and viral haemorrhagic fevers.Unless a high index of suspicion is maintained and laboratory assistance sought, clinical diagnosis is impossible, in majority cases and leptospirosis will be missed in all but a few instances.[2]

In an endemic area, suspecting leptospirosis on clinical grounds should not be very difficult. It is therefore necessary to increase the awareness and the knowledge of leptospirosis, as it is a public health threat

Dual infection of leptospirosis with other diseases such as Dengue[39] and Hepatitis B[40] have also been reported in places where these diseases are endemic and leptospirosis may be overlooked in such cases. Recognition of leptospirosis is especially important since antimicrobial agents can reduce its severity and duration.[85] A presumptive bedside diagnosis with serological confirmation may be made, only if a high index of suspicion is maintained.[40] Failure to identify leptospirosis in these patients will result in high mortality rate for this infection.

Hence this study was conducted with the objective of correlating different laboratory parameters including radiological features in leptospirosis so that a quick clinical suspicion can be made out and diagnosed early for initiating appropriate treatment and for a better outcome.

Review of Literature

REVIEW OF LITERATURE

HISTORY

Leptospirosis is a zoonosis of ubiquitous distribution caused by infection with pathogenic leptospira species.[46]

Adolf Weil described leptospirosis as a disease entity in 1886. His name is still attached to a serious form of leptospirosis called Weil's disease. traditionally attributed to rat transmitted infection, caused by the serovars *icterohaemorrhagiae* and *copenhageni*. At present, it is preferable to refer all leptospiral infections as leptospirosis regardless of clinical symptoms and signs. Goldsmidt first used the term Weil's disease in 1887. In 1907 Stimson demonstrated by silver staining the presence of clumps of spirochaetes that caused weil's disease in the kidney tubules of a patient who reportedly died of yellow fever. The spirochaetes had hooked ends and Stimson named them *Spirochaeta interrogans* because of their resemblance to question mark. Unfortunately this sentil observation was overlooked for many years.[20]

The etiology of leptospirosis was demonstrated independently in 1915 in Japan and Germany. In Japan, Inada and Ido detected both spirochaetes and specific antibodies in the blood of Japanese miners with infectious jaundice. In Germany, two groups of German physicians studied German soldiers afflicted by "French disease" in the trenches of Northeast France. Uhlenhuth and Fromme and Hubner and Reiter detected spirochaetes in the blood of guinea pigs inoculated with the blood of infected soldiers[46].

The first isolate made in 1917 from a patient in Japan with jaundice and haemorrhagic manifestations was named as Icterohaemorrhagiae. Subsequently other members of serogroups were isolated in various places of the world from different animals

reservoir hosts. Inada identified Leptospira *hebdomadis* carried by the field mouse as the causative agent for the nonicteric syndrome the “7 day fever”.[20]

ALTERNATIVE NAMES FOR LEPTOSPIROSIS

Autumnal fever, Cane field fever, Canicola fever, Field fever, Haemorrhagic jaundice, Mud fever, Rice Field fever, Seven Day fever, Bushy-Creek fever, Cane Cutter fever, European Swamp fever, Fort bragg fever, Pea Pickers Disease, Spirochaetosis, Stuttgart disease, Swamp fever, Swineherd disease, Wycon fever and Weil’s disease.

ETIOLOGY

Leptospirosis is an infectitious disease caused by pathogenic bacteria called leptospires. Leptospires are spirochaetes belonging to the Order Spirochaetales and Family Leptospiraceae.

DISTRIBUTION

Leptospirosis occurs worldwide but is most common in tropical and subtropical areas with high rainfall. The disease is found mainly wherever humans come into contact with urine of infected animals or a urine polluted environment.[94]

MORPHOLOGY

Leptospires are cork screw shaped bacteria which differ from other spirochaetes by the presence of end hooks.[94] Leptospires are long (6-20μm), thin (approximately 0.1μm)[22] tightly coiled spirochaetes, which are characterised by very active bending and rotating motility. Usually one or both ends are bent or hooked but straight forms also occur. The helical amplitude is approximately 0.1μm- 0.15μm and the wavelength is approximately

0.5μm.[21] Two axial filaments (periplasmic flagella) with polar insertions are located in the periplasmic space. The structure of the flagellar proteins is complex.[88] Morphologically all leptospires are indistinguishable, but the morphology of individual isolates varies with subculture in vitro and can be restored by passage in hamsters.[16] Leptospires pass through bacteriological filters of average pore diameter 0.2μm.[5]

Leptospires have a typical double membrane structure in common with other spirochaetes in which the cytoplasmic membrane and peptidoglycan cellwall are closely associated and are overlain by an outer membrane. Leptospiral lipopolysaccharides has a composition similar to that of other gram negative bacteria but has lower endotoxic activity.[74]

Though there are more than 260 serovars[41] of leptospira many of them are cross reactive due to the overlapping of the antigenic structure. Two types of antigen have been identified. The surface antigen contains protein polysaccharide and is serovar specific, while the somatic antigen contains genus specific lipopolysaccharide.[19] The outer membrane of the organism is a potent immunogen and is the target for the immunoglobulin- complement mediated bactericidal action.

Leptospira have a thermolabile oxygen stable, soluble hemolysin and various other enzymes like catalase, lipase, oxidase, peroxidase and hyaluronidase. Presence of endotoxin has also been shown in certain serovars. Neither bacteriophages nor plasmids have been isolated from leptospires. Leptospires are not known to produce bacteriocins.[21]

CLASSIFICATION

The genera Leptospira, Leptonema and Turneria belong to the family of Leptospiraceae. The families Leptospiraceae and Spirochaetaceae (genera Borrelia and

Treponema) make up the Order Spirochaetales. The classification and nomenclature of Leptospira is complex but presently two different classification systems are being used.[61]

1. Serologic Classification [Based on antigenic determinants]
2. Genotypic Classification [Based on genetic criteria]

1. Serologic classification

Prior to1989 the genus leptospira was divided into two species, L. i*nterrogans* comprising of all pathogenic strains and L. .*biflexa* containing the saparophytic strains isolated from the envoirrnment .[Faine et al., 1982, Johnson R. C. et al, 1984]. L.*biflexa* was differentiated from L. *interrogans* by the growth of the former at 13°C and growth in the presence of 8-azaguanine [225µg/ml] and by the failure of L.*biflexa* to form spherical cells in 1M Nacl.[20]

Both L. *interrogans* and L. *biflexa* are divided into numerous serovars defined by agglutination after cross absorption with homologous antigen (Dikken H et al., 1978).[15,35,41] Serovars are considered distinct if more than 10% of the homologous titre remains in atleast one of the two antisera on repeated testing.[31] Over 60 serovars of L. *biflexa* and over 200 serovars of L. *interrogans* are recognised.[31,32] Serovars that are antigenically related have traditionally been grouped into serogroups. Serogroups have no taxonomic standing, but the concept has proved useful for epidemiological purposes.

2. Genotypic classification

The phenotypic classification of Leptospires was replaced by a genotypic one, in which 17 genemospecies include all serovars of Leptospira.[9,67,95] DNA sequences of genes are attractive targets for phylogenetic studies. The sequence of the rrs gene, coding for 16s r RNA, is the most commonly used and accepted for studying genetic relationships (Perolat et

al., 1998).[65]

A classification system based on genetic traits should ideally allow subspecies characterisation. Typing methods should be simple to perform and give reliable results if they are to meet the needs of clinical and epidemiological practice.

A variety of methods of genetic analysis have become available in the last few years. The use of quantitative DNA-DNA hybridisation to measure DNA relatedness among leptospiral strains is the reference method of allocating strains to species.[94]

The genemospecies of leptospira do not correspond to the previous two species (L. *interrogans* and L. *biflexa*) and indeed, pathogenic and non-pathogenic serovars occur within the same species.[61]

Species based on genetic analyses are listed together with the serogroups most commonly present in these species.Neither serogroup nor serovar reliably predicts the true species of Leptospira.[61]

EPIDEMIOLOGY

(a) Agent factors

(i) Source of Infection

Leptospira are excreted in urine of infected animals for a longtime, often for an entire lifetime in cases of rodents.[63] Their alkaline urine pH and renal tissue pH are favourable for the organism's survival, permitting permanent colonising and urinal sheding.[8]

(ii) Animal reservoirs

Animals, including humans can be divided into maintenance hosts and accidental

(incidental) hosts. A maintenance host is defined as a species in which infection is endemic, usually transferred from animal to animal by direct contact. Infection is usually acquired at an early age and the prevalence of chronic excretion in the urine increases with the age of the animal. The animal hosts are not symptomatic and do not develop antibody despite overhelming infection.[23]

Other animals (such as humans) may become infected by indirect contact with the maintenance host. Animals may be maintenance host of some serovar, but incidental host of others, infection with which may cause severe or fatal disease.

The most important maintenance hosts are small mammals such as rats and small rodents which may transfer infection to domestic farm animals, dogs and humans. Different rodent species may be reservoirs of distinct serovars, but rats are generally maintenance hosts for serovars of the serogroup Icterohaemorrhagiae and mice are generally maintenance host for serogroup Ballum serovars fully. Domestic animals such as dairy cattle, pigs, dogs, sheep, cattle, goats, water buffalo also are maintenance hosts.[61] Infections may spread from wild animals to domestic livestock and then to humans.[60]

(b) Host factors

Human infection is usually due to occupational exposure to the urine of infected animals.

(i) High risk groups

Agricultural workers

Such as rice field planters, sugarcane and pineapple field harvestors, livestock handlers, labourers engaged in canal cleaning operations are subjected to exposure with leptospires which have reservoir in rodents, cattle, swine, sheep, goats etc.

Some other occupational groups are fishermen, sewer workers and all those persons

who are liable to work in rodent infested environment.

Lorry drivers and Masons

As lorry drivers may use contaminated water to wash their vehicles and Masons may come in contact with the organisms, while preparing the cement and sand mixture for construction work with contaminated water.

Leisure time activities such as swimming may also carry risks.

(ii) Age and sex distribution

Males suffer more frequently from leptospirosis than females because of greater occupational exposure to infected animals and contaminated environment. Gender difference in susceptibility is not apparent under conditions where both men and women are at equal risk. Leptospiral infections occur more frequently in persons 20-30 years of age group. Leptospirosis rarely occurs in young children and infants, possibly because of minimal exposure.[29]

(c) Environmental factors

Leptospirosis is acquired through contact with an environment contaminated by urine from carrier (reservoir) or infected animal or by handling of infected animal tissues.[73] Leptospirosis shed in urine can survive for weeks in soil and water, so environmental contamination may reach high levels in areas where carrier animals frequently urinate. The association of poor housing, limited water supply, inadequate method of waste disposal, all combine to make the disease a significant risk for the poor population in both urban and rural areas.[93]

Seasonal variation:

Leptospirosis peak incidence occurs in the rainy season or flooding after heavy rains in tropical regions and the late summer to early fall in temperate regions.[33,34,71,72]

MODE OF TRANSMISSION

(a) Direct contact

Leptospira can enter the body through skin abrasions or through intact mucous membrane by direct contact with urine or tissue of infected animal.

(b) Indirect contact:

Through the contact of broken skin with soil, water or vegetation contaminated by urine of infected animals.

Human to human transmission is rare. Man is usually a dead end host.[11]

CULTURAL CHARACTERISTICS

Leptospires are obligate aerobes. When cultivated in a suitable aerated medium at 30°C and an optimal pH of 7.2 to 7.6 their generation time varies from 7-12 hours and yields are 6 to 8×10^9 cells/ml. Leptospires cannot synthesise fatty acids de novo which is their main source of energy and carbon and also source for the cellular lipids. So in addition to long chain fatty acids, Vitamin B1, Vitamin B12 and ammonium salts(source for nitrogen) are also required for their growth. Leptospires incorporate purine bases but not pyrimidine bases into their nucleic acids and because of this they are resistant to the anti-bacterial activity of the pyrimidine analogue 5-Flurouracil.This compound is used in selective media for the isolation of leptospires from contaminated sources.[19]

Owing to the inherent toxicity of free fatty acids, these must be supplied to the leptospires either bound to albumin or in non toxic esterified form. Pyruvate enhances the initiation of growth of the parasitic leptospires. Leptospires can survive for many days to months in wet soil and fresh water with the pH in a neutral range. In salt water the survival

time is only few hours.[19]

CULTURE MEDIA

The type of media used for the isolation and cultivation of leptospires are media enriched with rabbit serum or Bovine serum Albumin(BSA) and protein free media. Liquid media are necessary for growing the cultures for serological diagnosis of infections and for typing the isolates. Liquid media are converted to a semi solid form by the incorporation of 0.2 percent agar and to the solid form by addition of 1% agar. Growth is readily initiated in these media and usually is easily visualised as one or more rings of dense growth several mm to cm below the surface of the medium, although a lack of rings of growth does not necessarily mean an absence of leptospires. Solid media are used for cloning the strains and for isolating leptospires from contaminated sources. Colonies in 1% agar are subsurface and become visible within 7-14 days.[7]

PATHOGENESIS

Once Leptospira gain entry in human body they spread through blood stream to all organs. Avirulent strains fail to multiply in the body and are removed from the blood in a day or two. Virulent leptospires multiply in the blood stream and in various organs and body fluids including the CSF. The organisms at this stage can be recovered from all tissues (Leptospiremic stage) for 4-7 days. Leptospires lead to extensive endothelial injury resulting in multiple haemorrhages throughout the body, transudation of fluid from the vascular compartment and hypovolemia.[21]

Agglutinating antibodies start appearing in the blood around the fifth day(Immune stage).[55] The organisms are then opsonized and removed from the blood by the reticulo-endothelial system. These agglutinating antibodies (IgM>IgG) are detectable by Microscopic

Agglutination Test(MAT).

After 4-7 days, the organisms persist in the aqueous humor of the eye, the renal tubules and are excreted in the urine. The Leptospiruria persists for 1-4 weeks in man.[21]

CLINICAL MANIFESTATIONS

Incubation period: Usually 7-12 days (range from 2-20 days).[70]

CLINICAL TYPES

The clinical spectrum of leptospirosis is very wide, with mild anicteric presentation at one end to severe leptospirosis with severe jaundice and multiple organ involvement on the other.[29]

On the basis of these clinical features, two types of leptospirosis are described.

Anicteric leptospirosis

- It is the milder form of the disease.
- Patients have fever, myalgia but do not have jaundice.
- Almost 90% of patients have this type of illness.

Icteric leptospirosis

- It is the severe form of the disease.
- It is characterized by jaundice and is usually associated with involvement of other organs.
- About 5-10% of patients have these type of manifestations.[66]

The clinical presentation of leptospirosis is **biphasic** and have two defined stages an initial Septicemic stage that is followed by an Immune stage. These stages are distinct in anicteric leptospirosis but not in icteric leptospirosis.[91]

ANICTERIC LEPTOSPIROSIS

1) SEPTICEMIC STAGE

This stage of the disease is abrupt in onset.[17] The patients present with :

• **Fever** -Patients have remittent fever with chills. It may be moderate to severe.

• **Myalgia**-It is a very characteristic finding in leptospirosis. Calf, abdominal & lumbosacral muscles are very painful & severely tender. This symptom is very useful in differentiating leptospirosis from other diseases causing fever.

There is associated increase in serum Creatinine Phosphokinase (C.P.K.) which helps in differentiating leptospirosis from other illnesses.[66]

• **Conjunctival Suffusion**-There is reddish colouration of conjunctiva. Very useful sign in leptospirosis. Usually bilateral, most marked on palpebral conjunctiva, it may be associated with unilateral or bilateral conjunctival haemorrhage.

• **Headache** -Usually intense, sometimes throbbing, commonly in frontal region. It is often not relieved by analgesics.

• **Renal manifestations** -Some form of renal involvement is invariable in leptospirosis. It usually occurs as asymptomatic urinary abnormality in the form of mild proteinuria with few casts & cells in the urine. Severe renal involvement in the form of acute renal failure, (which occurs in icteric leptospirosis) is rare.

• **Pulmonary manifestations** -Manifested in most cases through cough & chest pain and in few cases by haemoptysis. Severe involvement leading to respiratory failure does not occur in anicteric leptospirosis.

• **Hemorrhage**- Hemorrhagic tendencies are also present in some cases.

All the clinical features either decrease or disappear within two to three days and then they reappear.[29]

2) IMMUNE STAGE

The onset of immune stage coincides with the appearance of IgM antibodies. Aseptic meningitis is the hallmark of the immune stage.[6] The CSF cell count is <500/mm^3 in most cases. Polymorphonuclear cells may predominate early in the illness, but mononuclear cells predominate later. The CSF protein levels ranges from <40mg/dl (normal) to 300 mg/dl and the CSF glucose concentration is generally normal. Uveitis, iritis, iridocyclitis and chorioretinitis may also appear during the immune stage.[91]

• **Differential diagnosis**-The patients of anicteric leptospirosis are likely to be misdiagnosed as malaria, dengue hemorrhagic fever, viral hepatitis etc.

In endemic area all cases of fever with myalgia and conjunctival suffusion should be considered as suspected cases of leptospirosis.

ICTERIC LEPTOSPIROSIS: -(WEIL'S SYNDROME)

This is the more severe form of leptospirosis. As the name suggests all patients have jaundice. Patients present with: -

- Fever
- Myalgia
- Headache
- Conjuctival suffusion
- Oliguria/Anuria and/or proteinuria
- Nausea, vomiting
- Abdominal pain

In addition, they have features of organ involvement. An individual patient may have

features of one or more organ involvement. The more severe form of disease with severe liver and kidney involvement is known as Weil's syndrome. Salient features of these organ involvements are described below. [29]

Hepatic

Jaundice is the most important clinical feature. It may be mild to severe. It starts after 4 to 7 days of illness. Hepatic encephalopathy or death due to hepatic failure is rare. Hepatomegaly & tenderness in right hypochondrium are usually detected. Laboratory investigations show raised level of serum bilirubin (direct) and alkaline phosphatase. SGOT & SGPT are either normal or mildly elevated. This helps to differentiate leptospirosis from viral hepatitis where SGPT is markedly elevated and also from alcoholic hepatitis where SGOT is markedly elevated. High level of Creatinine Phosphokinase (CPK) is suggestive of leptospirosis. It is normal in viral hepatitis and alcoholic hepatitis. [29]

Renal

Renal involvement is almost invariably present in leptospirosis. In severe cases patients have acute renal failure and present with:

• Decreased urine output (oliguria or even anuria)

• Oedema may be present on face and feet.

• Features of uremia like breathlessness, convulsion, delirium and altered level of consciousness may be present in very severe cases.

The renal dysfunction worsens during the first week to the end of 2nd week, after which it starts improving and complete recovery occurs by the end of the 4th week. There is usually no residual renal dysfunction.[29]

Pulmonary involvement

High mortality due to pulmonary involvement is becoming a feature in Leptospirosis. There are wide variations in pulmonary presentation. It is the commonest cause of death due to leptospirosis.

Symptoms: In mild cases patient will show only cough, chest pain and blood tinged sputum. In severe cases patients have cough, haemoptysis, rapidly increasing breathlessness which may lead to respiratory failure and death. Leptospirosis presenting as adult respiratory distress syndrome has been described.[18,58] On examination, these patients have increased respiratory rate with basal creptations, which rapidly spread upwards to middle and upper lobes. X-ray shows basal and mid zone opacity in severe cases. It may be normal in mild cases. The under lying pathology is intra-alveolar haemorrhage.

More than ninety percent (90%) of deaths in cases of leptospirosis occur due to pulmonary alveolar haemorrhage.[29]

Cardiovascular system involvement

Patients can have any one or more of the following features:

1) Haemorrhage

They occur because of 1) Thrombocytopenia, 2) Disseminated Intra-vascular Coagulation (DIC), 3) Secondary to liver involvement leading to coagulation factor deficiency. Patients may have spontaneous superficial bleeding i.e. petechial, purpura, epistaxis or GIT bleeding. In severe cases ecchymosis or intra-cranial haemorrhage can occur.[29]

2) Hypotension Shock

Patient will have hypotension, cold clammy extremities, tachycardia, thready pulse. JVP is either normal or decreased. Echocardiography reveals normal systolic function of left ventricle hence hypotension is due to either dehydration or peripheral vasodilatation.

3) Arrhythmias

Patient presents with palpitation and syncope & irregular pulse. Common arrhythmias seen are supraventricular tachyarrythmias and various degrees of A.V. blocks. Ventricular tachyarrhythmias are infrequent. ST Segment depression and T wave inversion may be present in some patients.[29]

SUMMARY OF ORGANS AFFECTED IN ICTERIC LEPTOSPIROSIS:

Organ	Clinical features	Investigations reveal
Kidney	Decrease in urine output, Features of uremia	Increase in Serum Creatinine, Increase in Blood Urea
Liver	Jaundice, hepatomegaly	Increase in Serum Bitirubin with Normal or midly elevated SGPT and SGOT and increased CPK
Lungs	Cough, haemoptysis, dyspnoea with increase in respiration rate and basal creps	X ray chest shows lower and mid zone opacities
Heart	Hypotension, Irregular pulse	ECG reveals the type of arrhythmia
Blood	Bleeding tendencies	Decrease in platelet count
Brain	Altered consiousness with Neck rigidity	CSF shoes increase in cells, increase In protein, normal sugar

(Guidelines WHO 2006)

LABORATORY DIAGNOSIS OF LEPTOSPIROSIS

The diverse clinical presentations of this disease make it essential for the laboratory to play a role in diagnosis. Microbiological diagnosis of leptospirosis aims at demonstrating the leptospires, by culturing them or by demonstrating an appreciable antibody response to them.[7]

DIRECT EVIDENCE(ANTIGEN DETECTION TESTS)

1. Demonstration of leptospires or their products:

Microscopy

- Dark-field microscopy
- Phase contrast microscopy

Staining

- Silver staining
- Immunofluorescence
- Immunoperoxidase

DNA hybridisation

Polymerase chain reaction

Random Amplified Polymorphic DNA Fingerprinting (RAPD)

2. Isolation of leptospires

Blood

Urine

CSF

Body fluids and tissues

3. Animal Inoculation

INDIRECT EVIDENCE

1. Detection of antibodies to leptospira

1) Genus specific tests

- Macroscopic agglutination test (MSAT)
- Indirect fluorescent antibody test (IFAT)
- Indirect haemagglutination test (IHA)
- Counter immuno electrophoresis (CIEP)
- Complement fixation test (CFT)

Newer techniques

- Enzyme Linked Immuno Sorbent Assay (ELISA)
- Microcapsule agglutination test (MCAT)
- Lepto-Dipstick
- Lepto lateral flow test
- Lepto DRI Dot

2) Serogroup/serovar specific tests

- Microscopic agglutination test (MAT)

Collection of sample

Leptospires may be present in the blood and CSF in the first 7-10 days of illness and in the urine from the 10-14 day of illness to 30 day of illness. Aggulinating antibodies appear in the blood towards the end of the first week reaches the peak in the third or fourth week of illness and then gradually decline with low levels persisting for an indefinite period.

I. Demonstration of Leptospires or their products.

Dark-Field Microscopy

The typical motility of the leptospires in the clinical sample (blood, CSF, urine or peritoneal fluid) observed with dark-field microscopes, when correlated with clinical parameters, may aid in early diagnosis. It is a simple method, but it may not be positive if there are few bacteria in the sample. Double centrifugation of the sample at low speed to separate the cellular elements, and then at high speed, is needed to help concentrate the leptospires. Artefacts like lysed RBCs, fibrils, etc. may however, be mistaken for leptospires. So it is not recommended as the only diagnostic procedure to be used .[7]

Phase contrast microscopy is useful for visualizing leptospires in the laboratory, but, because of its technical limitations in thick suspensions and its optical characteristics, it has no practical purpose whenever dark-field microscopy is available .[19]

Staining Methods

Silver deposition techniques:

Leptospires in smears of tissues or fluids on slides can be stained using silver deposition methods. The variously described procedures are modifications of Warthin Starry's method for staining. The stain is based on chemically reducing surface properties of leptospires and other spirochaetes. Well-stained preparations show black spirochaetes in pale yellow or brown tissue elements. This method has the same limitation as dark-field procedures, as it is difficult to detect small numbers of organisms in tissue sections, and artefacts may be mistaken for leptospires .[19]

Immunofluorescence

Immunofluorescence staining of leptospires is often preferable to silver staining because it is easier to see leptospires, especially in small numbers, and the serovars or serogroups can be determined presumptively. When a combination of antisera labelled with different fluorochromes is used, more than one serological type of leptospires can be identified in the same preparation. One disadvantage is the need for special fluorescent microscopy equipment; another is that specially prepared labelled antisera are required. A double layer or sandwich method is used with primary specific anti leptospiral antisera and a secondary universal fluorochrome labelled anti rabbit globulin serum.[70]

DNA hybridisation

Leptospira specific DNA sequences can be hybridised to leptospiral DNA extracted from clinical specimens. The reaction can be visualised with the help of an avidine biotinylated peroxidase complex plus a colour reagent. This technique is not very sensitive.

Polymerase chain reaction (PCR)

PCR involves in vitro enzymatic amplification of a target DNA sequence through a series of polymerisations carried out by a thermostable DNA polymerase, primed with a pair of the short DNA fragments, which bind specifically to the sequence of interest. Amplified DNA fragments produced by this technique can be easily visualized on ethidium-bromide-

stained agarose gels with a UV transilluminator.[67]

Random amplified polymeric DNA (RAPD) finger printing

This is another molecular typing method based on PCR amplification and finger printing, using arbitrary oligonucleotide primers, which produces reliable and easily obtainable typing schemes[67].

2) Culture of leptospira

The infecting strains can often be isolated in culture, provided that suitable material is obtained before antibiotics have been administered. Early in the course of illness – during the leptospiraemic phase – the inoculum of preference is blood or cerebrospinal fluid; later during the phase of leptospiruria – it is urine. It is particularly valuable in man, as the serological response can be slow and may be absent altogether if antibiotics are given early. Since serology is usually serogroup specific, isolation is essential to identify the infecting serotype. Such information is essential for epidemiological purposes, for the selection of relevant leptospires for use in diagnostic tests, vaccine preparation and for the assessment of antibiotic sensitivity.[70]

3) Animal inoculation

Laboratory animals are useful for isolating the organisms from contaminated materials and for maintaining recent isolates. They are also sometimes essential for decontaminating cultures, and with the help of passive protection, they may be used to recover a single

serotype from a mixed culture. Young animals, preferably weanlings, should be used because older animals may resist the infection. Stocks must be free from endemic leptospiral infection; guinea pigs, hamsters, gerbils, young rabbits, swiss white mice, albino American deer mice and 1 to 3 day-old chicks may be used. The material should be inoculated intraperitoneally through one of the lower quadrants of the abdominal wall. The animals should be examined twice daily, and a drop of peritoneal fluid can be examined with dark-field microscopy for active leptospires from the 3rd to the 7th day.[75]

II. Serological Methods(Detection of antibodies to leptospira)

Serological tests can be a guide to the infecting serum and this information is useful for prognosis and epidemiology. Serological tests do not react until a few days after infection, but reactions persists for months or years. Persistent antibodies allow retrospective diagnosis,[21] but seroconversion or a 4-fold or greater rise in titre in paired serum samples in the presence of a compatible clinical illness is an important criterium for the definitive diagnosis of leptospirosis. The wide range of tests that are available are broadly divided into genus-specific and serogroup/serotype-specific tests.

1) Genus Specific Tests

The antigen for these tests is prepared from the non-pathogenic L. biflexa Patoc – 1 strain.

1. MACROSCOPIC SLIDE AGGLUTINATION TEST (MSAT)

A rapid macroscopic slide agglutination test can be used to screen serum samples.

These tests are carried out with a dense suspension of leptospires, which agglutinate into clumps visible to the naked eye. The best method is Galton's macroscopic slide agglutination test, in which 12 antigens were originally proposed, and later supplementary antigens were suggested.[25] MSAT is found to be a simple, rapid, and sensitive diagnostic test for active leptospirosis; the sensitivity of the test can be improved by the addition of locally prevalent serovars.[83] This test is slightly less specific than MAT, but it gives a positive reaction earlier in disease.

2. INDIRECT IMMUNOFLUORESCENCE TEST

In 1966, Patoc strain 1 was used in an indirect immunofluorescence test. Immunofluorescence has not been used widely for primary diagnostic tests. It is used to detect leptospirosis in tissues.[20] This is a fast and reliable test, where facilities are available. It has a sensitivity of 91.4%.[7]

3.INDIRECT HAEMAGGLUTINATION TEST (IHA)

This is a genus specific test where the broadly reactive antigen is coated on erythrocytes. This test can be done in microtitre plates or tubes.

4. COUNTER IMMUNO ELECTROPHORESIS(CIEP)

The patient serum and antigen are allowed to move under the influence of electric current through a gel. Presence of antibodies can be detected by a visible precipitate.

5. COMPLEMENT FIXATION TEST(CFT)

It is a complicated technique and is not used in diagnostic laboratories.

6.ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

This is for the detection of IgM antibodies that has been shown to be more sensitive than MAT when the sample was taken early in the acute phase of the illness. IgM antibodies have been detected by ELISA in CSF from patients with icteric Leptospirosis. ELISA methods have been applied with a number of modifications.

An IgM specific dot ELISA was developed in which polyvalent leptospiral antigen was dotted on to nitrocellulose filter discs in microfilter tray wells, allowing the use of smaller volumes of reagents. Further modifications of this approach have been used to detect IgG and IgA in addition to IgM tests. These tests have employed an immunodominant antigen and a polyster fabric resin support in place of nitrocellulose. A Commercial IgM-dot ELISA dipstick has been shown to be as sensitive as microtitre plate IgM-ELISA.[3]

7. MICROCAPSULE AGGLUTINATION TEST (MCAT)

This test was developed for serodiagnosis of Leptospirosis, based on the passive agglutination of synthetic polymer carriers, sensitized with mixed antigens of sonicated leptospires, by leptospiral antibody.. The MCAT gave positive results earlier in the course of the disease but may not detect antibodies in sera collected more than 1 to 2 months after the

onset of disease.[75]

8. LEPTO DIPSTICK

This dipstick assay is for the detection of leptospira-specific IgM antibodies in human sera Heat stable antigen prepared from *Leptospira biflexa* and coated onto the lower band and internal control is set up in the upper band; detection agent is also incorporated. The strength of staining is important in interpretation of test results. This test is simple to perform and easy to read but expensive.

9. LEPTO LATERAL FLOW TECHNIQUE

It is a rapid colloidal gold immunoassay technique in which the antibody in the serum which is added on to the vent permeates along nitrocellulose membrane picks up the conjugate and reacts with antigen impregnated on the test band and produces a colour which is compared with a given positive control band.

10. LEPTO DRI - DOT ASSAY

This assay consists of coloured latex particles activated with broadly reactive leptospiral antibody that is dried onto an agglutination card. This assay is based on the binding of leptospira specific antibody present in the serum sample to the antigen causing a fine granular agglutination that tends to settle at the edge of the droplet.[80]

2) Serovar/Serogroup specific Tests

1. MICROSCOPIC AGGLUTINATION TEST (MAT)

The MAT is slow, tedious, potentially biohazardous, painstaking and subjective; but it is a very sensitive and reliable assay. MAT is carried out with suspensions of live cultures or of cultures killed by the addition of formaldehyde. The clumps of agglutinated living leptospires differ in appearance from clumps of killed cultures. Living leptospires are agglutinated into highly refractile spheroids of various sizes, some of which may be joined to produce elongated masses of confluent spheroids. By contrast, the agglutinated killed leptospires form looser masses with an irregular often angular, outline; these appear flattened, resembling small piles of threads, or snowflakes, or pieces of cotton wool. The degree of agglutination ranges from 100%, when no free leptospires can be seen between the clumps, through lesser degrees, as the serum is more diluted, to nil, as seen in the negative control suspension of leptospires in diluent. The degree of agglutination can only be assessed in terms of the proportion of free leptospires. The accepted endpoint of an agglutination reaction is the final dilution of serum at which 50% or more of the leptospires are aggluatinated.[89]

Interpretation of diagnostic MAT: In a non-endemic area any level of antibodies, however low, may signify leptospirosis in the 1st week of a clinically compatible illness. The titer will rise in a second specimen taken after 3 to 7 days. If the titer remains below 100, even on repeated testing, it may be assumed that it was due to previous leptospirosis, and not to current illness. A titre of 400 to 800 or more, or a 4-fold rise in titer between 2 tests, is diagnostic when combined with a clinical illness compatible with leptospirosis. In endemic areas, the diagnosis will be confirmed if the titre rises on retesting, but will be negated if it is unchanged, assuming that the infecting serovar was included among the antigens for the

MAT.[22]

LEPTOSPIROSIS WITH CONCOMITANT ILLNESSES

Leptospirosis can occur with other infections as dual infection in the same patient.[3]

Dengue

Concurrent epidemics of leptospirosis and dengue fever because of their epidemiological similiarities have been reported.[24,38,44] Kaur and John reported dual infection with leptospira and dengue fever virus[39]. In the year 2002, in Mumbai concurrent outbreaks of leptospirosis and dengue occurred.[86] In a study at Brazil, Ko Al Galvao Reis (1994) reported misdiagnosis of leptospirosis as dengue in the outpatient clinic.[42] During the second half of 1995, an epidemic of degue type I infection occurred. In that period, leptospirosis mortality was twice the average, suggesting that some cases of leptospirosis were being misdiagnosed and treated inappropriately.[47]

HepatitisB

Weil's syndrome characterised by jaundice, renal failure and bleeding manifestations is the most severe form of leptospirosis. Weil's syndrome and concominant Hepatitis B infection infection have been reported by Kaushik and Colleagues.* Weil's disease is likely to be misdiagnosed or overlooked in patients with chronic Hepatitis B virus infection due to potentially overlapping clinical features.[45] A high index of suspicion for Weil's syndrome resulted in the prompt use of high dose of Penicillin and the patient recovered from

leptospirosis despite the severe systemic manifestations of leptospirosis and combined hepatic involvement of these two illnesses.[40]

Dual infection with Hanta virus, Hepatitis E virus, Malaria parasites, Herpes Simplex virus, Scrub typhus and coinfection with three different pathogens has also been reported but rare.[4,43,87]

TREATMENT

Penicillin, Streptomycin, Tetracycline, Erythromycin and cephalosporins are the antimicrobials capable of killing leptospires. Early institution of antimicrobial treatment may prevent complications like renal failure. Parenteral aqueous benzyl penicillin in a dosage of 1 mega unit 6 hourly for a period of 1 week is recommended. For patients with penicillin hypersensitivity, tetracycline at a dosage of 2g daily is recommended provided renal function is normal. Doxycycline (200 mg daily) and third generation cephalosporin like cefataxime or ceftriaxone given intravenously are also found to be highly effective.

PREVENTION

Prevention consists of avoiding direct or indirect exposure of humans to infected animal urine and tissues. Occupational hygiene, protective clothing and safe work practices are important in preventing the disease in working population. Vaccines are effective in preventing the disease in animals. The outer membrane vaccine is more effective than the whole cell and protoplasmic cylinder vaccine. Its use on man is impracticable due to the presence of many serovars. However vaccination against a specific serovar prevalent in the

area has been shown to be effective. Chemoprophylaxis with Doxycycline 200mg weekly prevents infection but is recommended only for short term use in high risk individuals.[21]

Aims of the Study

AIMS OF THE STUDY

- To study and correlate the haematological, bio-chemical and radiological parameters in the diagnosis of leptospirosis.
- To identify the predominant serovar in leptospirosis
- To study the concomitant infection of leptospirosis with Dengue and Hepatitis B

Materials & Methods

MATERIALS AND METHODS

STUDY PERIOD

March 2007- February 2008

PLACE WHERE STUDY WAS CONDUCTED

Leptospirosis Research Cell (LRC), Institute of Microbiology, Madras Medical College(MMC), Chennai-3.

STUDY POPULATION

This cross sectional study included 378 patients who attended Government General Hospital, Chennai with fever of 5 days and more duration and of the age group 11 years and above. Only patients with features suggestive of leptospirosis were included in this study. Patients with fever associated with malignancies, autoimmune diseases were excluded from this study.

ETHICAL CONSIDERATION

Written consent to participate in the study was obtained from the patients or their guardians after providing the full explanation of the study to them. This study was reviewed and approved by Institutional Ethical Committee, Madras Medical College and General Hospital, Chennai – 3. All datas were handled confidentially and anonymously.

INSTITUTIONAL ETHICAL COMMITTEE APPROVAL NUMBER

K.Dis.No.16328 P & D3/Ethics/Dean/GGH/08.

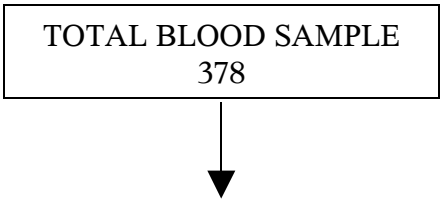
STATISTICAL ANALYSIS

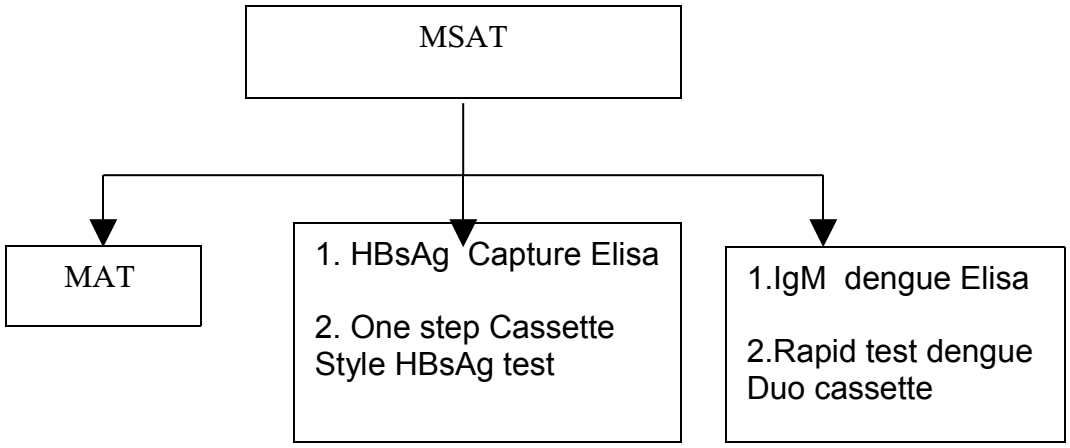
Demographic and clinical variables were given in frequencies with their percentages.Incidence were given in proportion with 95% confidence interval. Statistical difference were found using one sample chi-square test. $p<0.05$ were considered statistically significant.

MATERIALS

- 378 blood samples were collected from patients with clinical symptoms suggestive of leptospirosis during this study period.
- From all these patients 2ml of blood was collected by venepuncture with aseptic precautions and the serum was subjected to serological test for leptospirosis – MSAT.
- 156 sample out of this were positive for the serological test MSAT.
- Detailed clinical history was obtained from all positive cases of leptospirosis.
- Biochemical profile, haematological parameters and radiological parameters of the leptospirosis positive patients were studied.
- 10 ml of blood was collected from these patients, serum separated and investigated for
    1. Dengue IgM, antibody capture ELISA,
    2. Rapid test - Dengue Duo cassette.
    3. HBs Ag capture ELISA.
    4. Rapid test – One step Cassette Style HBsAg Test

ALGORITHM OF TESTING PROCEDURE





METHODS

Leptospiral serovars *autumnalis, australis, icterohaemorrhagiae, Louisiana, grippotyphosa, hebdomadis canicola semaranga* were used for MSAT and MAT. The above standard research strains were obtained from KIT and Royal Tropical Institute, Netherlands and subcultured periodically in LRC, Institute of Microbiology, MMC, Chennai-3.

MEDIUM

EMJH (Ellinughausen – Mccullough – Johnson – Harris) medium was used for subculturing the organism for maintenance and antigen preparation.

MACROSCOPIC SLIDE AGGLUTINATION TEST (MSAT)

(Mazzonelli et al 1974)[52]

- Different serovars of leptospira were grown in EMJH liquid medium for 7 days at 30ºC in a shaking incubator.

- After checking for growth and purity the leptospires were killed in formalin,(0.5ml of formaldehyde in 100ml culture).
- After 30mts the killed leptospira culture was kept in boiling water bath for 30 mts.
- The culture was rotated at every 15 mts
- After cooling to room temperature, the culture was centrifuged for 30mts at 10,000 rpm.
- .The supernatant was used as antigen. A pooled suspension of locally prevalent serovars namely *icterohaemorrhagiae, autumnalis, australis, louisiana, grippotyphosa, hebdomadis,canicola and semaranga* were used as antigens in MSAT.

PREPARATION OF PHOSPHATE BUFFER SALINE (PBS)(Ph 7.2)

NaCl	8gm/litre
K_2HPO_4	1.12gm/litre
KH_2PO_4	0.34gm/litre
Distilled water	1 litre

PROCEDURE

- 8µl of PBS was added to all the depressions of the slide
- Then 11µl of the prepared antigen was added to all the depressions of the slide
- Negative serum added with antigen and PBS was used as negative control
- Positive serum added with antigen and PBS was used as positive control

- The last depression containing saline and antigen was used as antigen control
- 4ml of patients serum was added to the depressions in the slide
- The slide was placed in a rotator (180rpm) for 8 mts
- The slide was viewed macroscopically for the presence of any agglutination clumps and was confirmed in DFM
- The results of MSAT were read as follows:

1. Clumps of Agglutination with complete clearing of leptospiral antigen	4+
2. Obvious Agglutination with partial clearing of antigen suspension	3+
3. 50% Agglutination	2+
4. 25% Agglutination	1+

No agglutination and uniformity of serum antigen mixture-Negative.

INTERPRETATION OF RESULTS

An agglutination of ≥2 is considered as positive for leptospirosis.

MICROSCOPIC AGGLUTINATION TEST

(Sulzer and Jones 1976) [82]

The MAT was performed with eight (8) live cultures using standard microtitre methodology.

S.NO	ANTIGEN PANEL
1.	L .*icterohaemorrhagiae*
2.	L .*australis*
3.	L .*autumnalis*
4.	L .*hebdomadis*
5.	L .*grippotyphosa*

6.	L .*louisiana*
7.	L .*canicola*
8.	L .*semaranga*

The sera were initially screened at dilution of 1:20 and those that were positive were titrated further upto the end point. The highest dilution of serum that agglutinated 50% of leptospires under dark field microscopy were presumed to represent the titre of antibody specific for the particular serogroup used. When two or more serogroups react at the same (highest) titre, the result was recorded as mixed equal. Controls were put up for each one of the battery of the antigens used in the test.

PROCEDURE

The sera diluted to 1:10 in separate test tubes.

- 25µl of PBS was added to all the wells in the microtitre plate
- The sera was diluted by doubling dilution as 1:20, 1:40 upt0 1:320
- Last well serves as a control containing only PBS and antigen
- 25µl of antigen was added to each well in the row(Each row of the microtitre plate was used for serovar from the panel)
- The serum antigen mixture then mixed by gentle agitation and it was kept at room temperature for 2 hrs
- One drop from each well was examined from lower dilution onwards after examining the antigen control
- By dark field microscopy, agglutination masses and free organisms were looked for.

INTERPRETATION OF RESULTS

The highest dilution of serum antigen mixture, which showed 50% agglutination was taken as the end point titre of the serum for that particular antigen.

CRITERIA FOR DIAGNOSIS

An initial titre > 1.80 or a four fold rise in titre of MAT was considered as positive.

DENGUE IgM CAPTURE ELISA: (PANBIO)

Detection of IgM antibodies to dengue virus by ELISA is a valuable procedure. The IgM antibodies appear as early as 3 to 5 days after the onset of fever and generally persist for 30 to 90 days.

Principle: (Appendix iii).

PROCEDURE

1. Dilute the antigen 1/250 using the antigen diluent.
2. The required amount of diluted antigen is mixed with an equal volume of MAb tracer in a plastic vial.
3. The antigen-MAb tracer solution was gently mixed and left at room temperature.
4. Within 10 minutes after mixing the MAb tracer and diluted antigen, pipette 100µl diluted patient samples into their respective microwells of the assay plate leaving A_1 to E_1 wells.
5. In A_1 positive control serum in B_1 negative control serum and in C_1 , D_1 , E_1 cut off calibrator serum were added.

6. Cover the plate and incubate for 1 hour at 37°C± 1°C.
7. Wash 6 times with diluted wash buffer.
8. Mix the antigen-MAb tracer solution before transfer pipette 100µl of antigen-MAb complexes from the antigen vial to the appropriate wells of the assay plate.
9. Cover the plate and incubate for 1 hr at 37°C.
10. Wash 6 times with diluted wash buffer.
11. Pipette 100µl of stop solution to all wells in the same sequence and timing as the TMB addition. Mix well. The blue colour will change to yellow.
12. Within 30 minutes read the absorbance of each well at a wavelength of 450nm with a reference filter of 600 to 650 nm.

CALCULATIONS

Average absorbance of the triplicates of the cut off calibrator = cut off value.

$$\frac{\text{Sample absorbance}}{\text{cut off value}} = \text{Index value}$$

(OR)

Index value × 10 = Panbio units

INTERPRETATION OF RESULTS

Index	**Panbio units**	**Result**
< 0.9	< 9	Negative
0.9 – 1.1	9 – 11	Equivocal
> 1.1	> 11	Positive

DENGUE DUO CASSETTE

This is a rapid test done for the qualitative presumptive detection of IgM and IgG antibodies to dengue virus in human serum, plasma or whole blood.

TEST PROCEDURE

- 10µl of serum was added to the sample well using a micropipette.
- 2 drops of buffer was added to the square well at the base of the cassette.

The result were read exactly 15 minutes after adding the buffer to the cassette. Any trace of a pink line in the test area indicated a positive result.

INTERPRETATION OF RESULTS

1. Primary infection: Pink bands appeared in the IgM and control regions.
2. Secondary infection: Pink bands appeared in the IgM, IgG and control regions or on the IgG and control regions.
3. Negative result: Pink bands appeared in the control region. No bands in the IgM or IgG regions.
4. Invalid test: No band appeared in the control region.

HBsAg ELISA TEST KIT – (HEPALISA)

PRINCIPLE

This is a solid phase enzyme linked immuno sorbent assay (ELISA) based on the ‘Direct Sandwich” principle.

PROCEDURE

- Leave A-1 well as blank.
- Add 50 µl Negative control in each well No. B-1 and C-1 respectively.
- Add 50 µl Positive control in D-1, E-1 and F-1 wells.
- Add 50 µl of sample in each well starting from G-1.
- Add 50 µl of working Enzyme conjugate to each well except A-1. Gently shake the plate for 2 to 3 seconds to mix the sample and conjugate.
- Cover the plate and incubate in an incubator at 37°C ± 1°C for 60 minutes.
- Dilute the wash buffer concentrate with distilled water to 1:25 dilution.
- At the end of incubation period, take out the plate from incubator and wash with working wash buffer.
- Tap dry the wells after washing and add 100 µl od working substrate solution in all the wells including A-1.
- Cover the plate with an aluminium foil and incubate at room temperature (20-25°C) for 30 minutes in dark.
- Stop the reaction by adding 50 µl of stop solution to each well, mix gently.
- Read the absorbance of the wells at 450 nm in an Elisa Reader after blanking A-1 well. (Use of a reference filter of 630 nm is preferred).

CUT OFF CALCULATION

Cut off value = NC mean + 0.1

where NC mean is mean absorbance(O.D) of Negative Control.

INTERPRETATION OF RESULTS

The absorbance of the unknown sample is compared with the calculated cut off value.

- Test specimens with absorbance (O.D) value less than cut off value are non-reactive and may be considered as negative for HBsAg.
- Test specimens with absorbance (O.D) value greater than or equal to cut off value are reactive for HBsAg.

ONE STEP CASSETTE STYLE HBsAg TEST

This is a rapid, direct binding test for the visual detection hepatitisB surface antigen (HBsAg) in serum based on the principle of sandwich immunoassay.

TEST PROCEDURE:

- About 3 – 4 drops of sample was added onto the sample well of the cassette.
- Wait for 10 – 20 minutes and read results.

INTERPRETATION OF RESULTS

1. **Negative result:** Only one colored band appears on the control (C) region. No

apparent band on the test (T) region.

2. **Positive result:** In addition to a pink colored control (C) band, a distinct pink colored band will appear in the test (T) region**.**
3. **Invalid Test:** A total absence of color in both regions or no colored line appears in the control (C) region.

Results

RESULTS

Total No of samples taken : 378

Total No of MSAT positive samples : 156

Total No of samples included in the study : 156

TABLE 1

MACROSCOPIC SLIDE AGGLUTINATION TEST

Total samples	Procedure	No. of positive cases	Percentage
378	MSAT	156	41.26

41.26% of patients were positive by MSAT. 95%

Confidence Interval is 36% - 46%.

TABLE 2

AGE DISTRIBUTION n=156

Age	No. of Cases	Percentage
11-20	21	13.46
21-30	37	23.71
31-40	31	19.87
41-50	30	19.23
51-60	23	14.74
>60	14	8.97

The incidence was relatively higher in 21-30 age groups

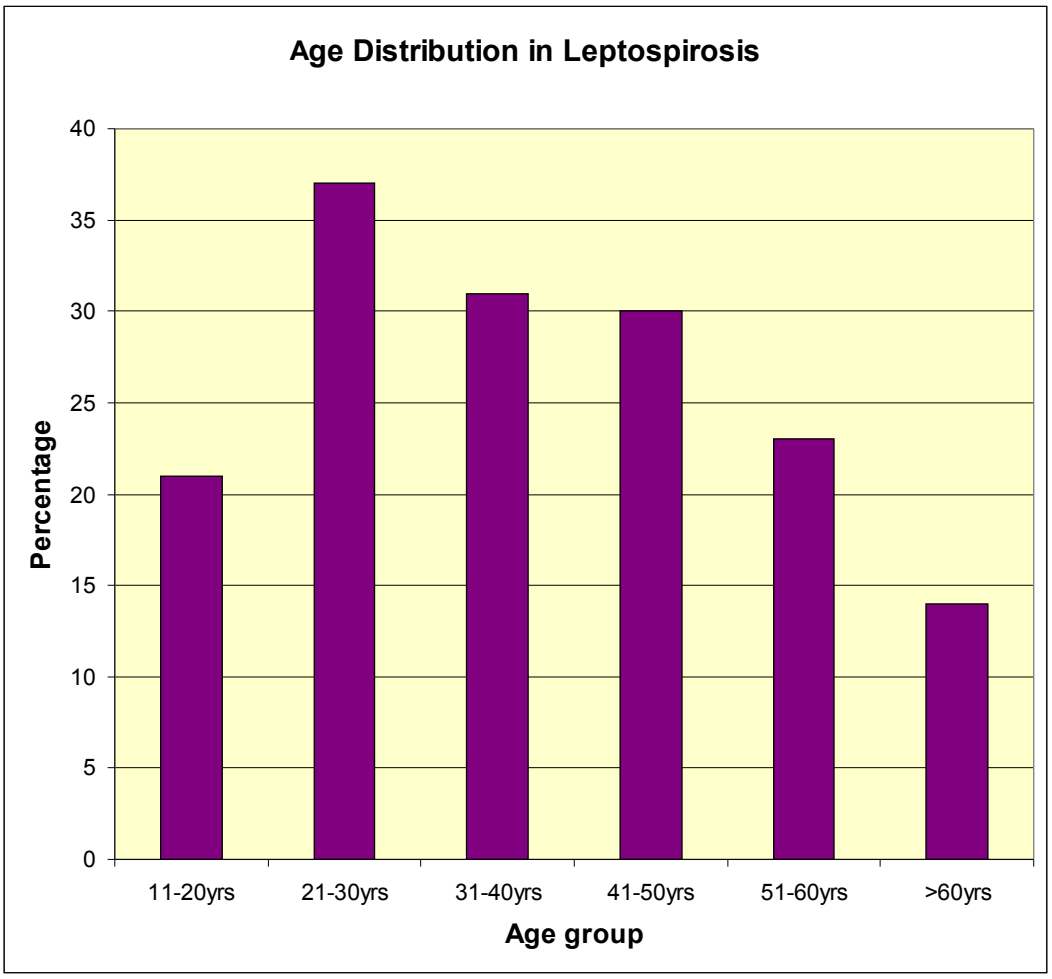

Age Distribution in Leptospirosis
Percentage
40
35
30
25
20
15
10
5
0
11-20yrs
21-30yrs
31-40yrs
41-50yrs
51-60yrs
>60yrs
Age group

TABLE 3

SEX DISTRIBUTION n=156

Gender	No. of cases	Percentage
Males	80	51.28
Females	76	48.71

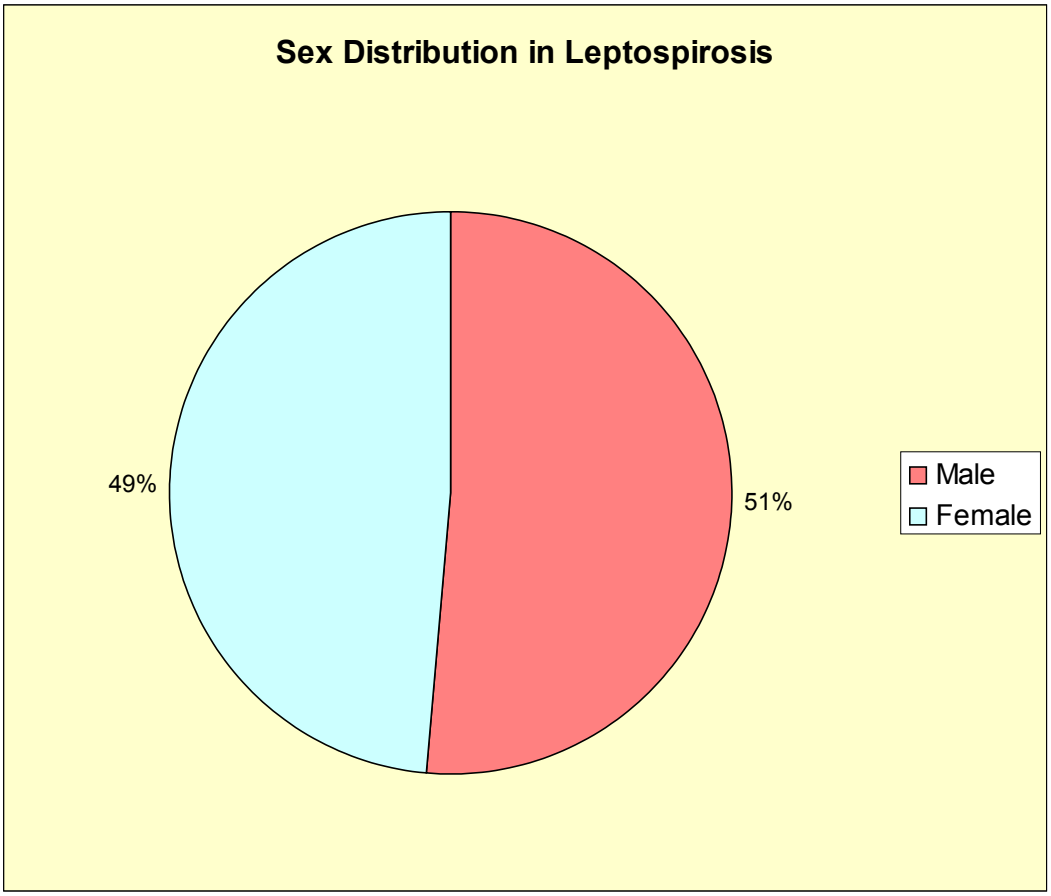
There was almost equal incidence of leptospirosis in males and females with very slight preponderance in males. One Sample Chi-square test $x^2=0.10, p=0.75$.

TABLE 4

SEASONAL DISTRIBUTION OF INCIDENCE OF LEPTOSPIROSIS n=156

Month	No. of cases	Percentage
March '07	11	7.05
April '07	10	6.41
May '07	12	7.69
June ' 07	10	6.41
July '07	13	8.33
August '07	14	8.97
September '07	15	9.61
October '07	13	8.33
November '07	14	8.97
December '07	15	9.61
January '08	15	9.61
February '08	14	8.97

Incidence of leptospirosis was seen throughout the year, although the number of cases increased after rainy season.


Sex Distribution in Leptospirosis
49%
51%
Male
Female

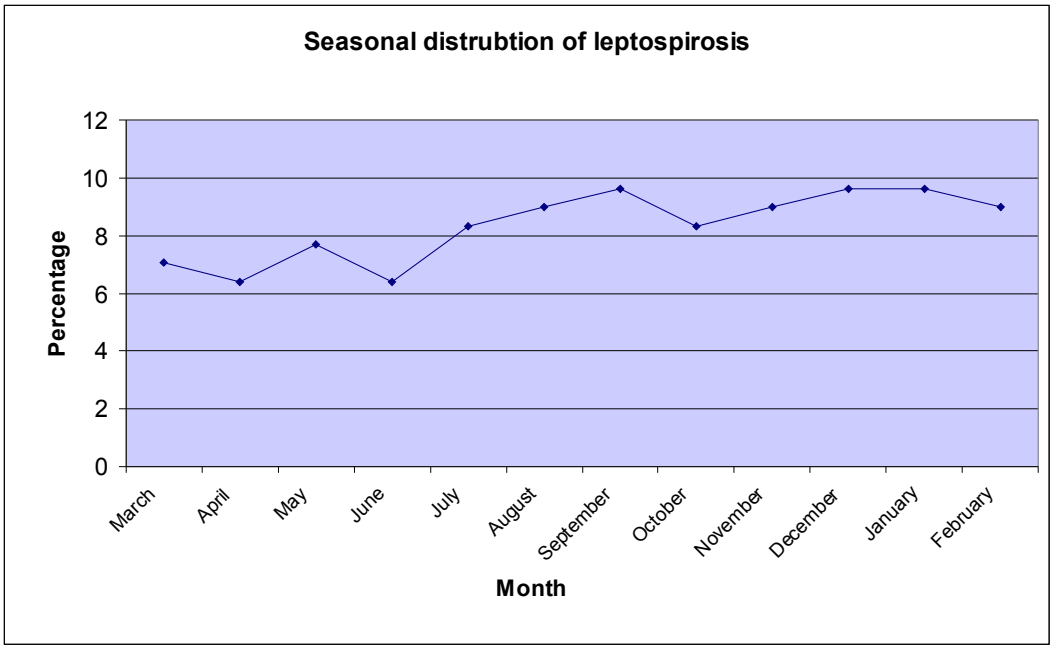

Seasonal distrubtion of leptospirosis
Percentage
12
10
8
6
4
2
0
March
April
May
June
July
August
September
October
November
December
January
February
Month

TABLE 5

SYMPTOMS OF CASES PRESENTING WITH LEPTOSPIROSIS n=156

Symptoms	No. of cases	Percentage
Fever	156	100
High coloured urine	98	62.82
Vomiting	75	48.07
Headache	63	40.38
Abdominal pain	54	34.61
Diarrhoea	53	33.97
Cough	53	33.97
Myalgia	50	32.05
Breathlessness	31	19.87
Pedaloedema	18	11.53
Conj. Suffusion	17	10.89
Joint pain	16	10.25
Dysuria	13	8.33
Hemoptysis	9	5.76
Giddiness	8	5.12
Oliguria	8	5.12
Restlessness	7	4.48
Haematuria	7	4.48
Altered sensorium	7	4.48
Skin rashes	5	3.20

Fever was the predominant symptom followed by high coloured urine, vomiting, headache.

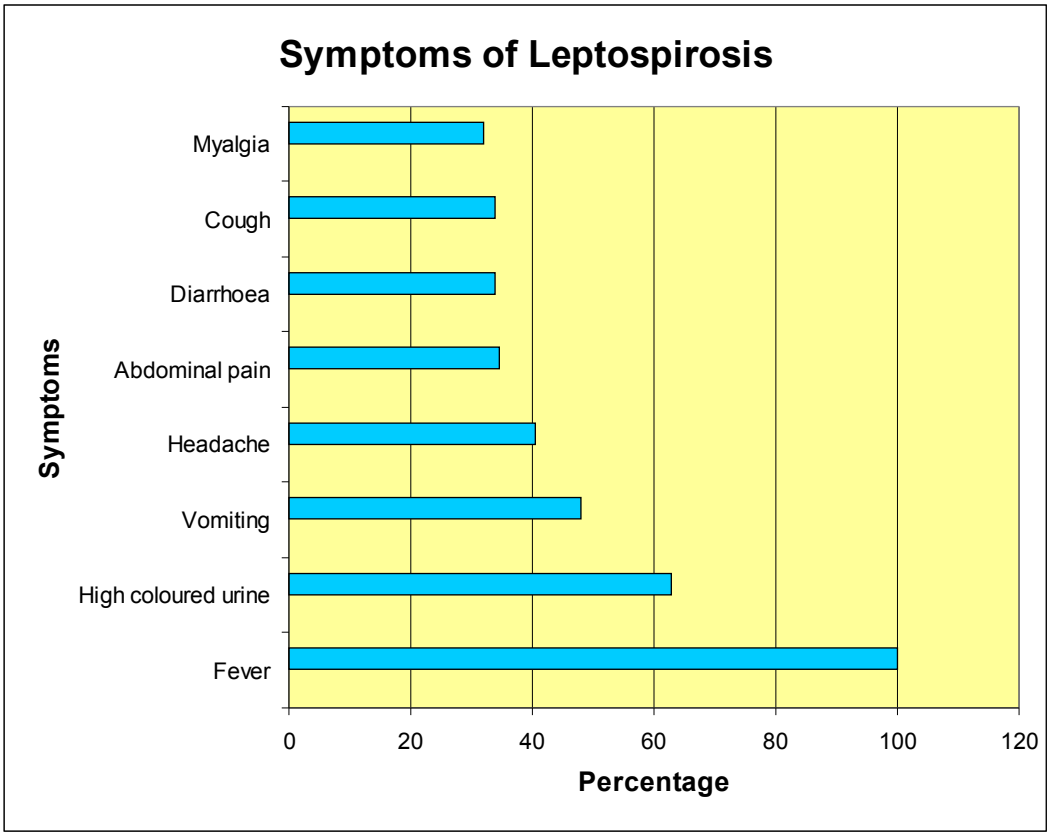

Symptoms of Leptospirosis
Symptoms
Myalgia
Cough
Diarrhoea
Abdominal pain
Headache
Vomiting
High coloured urine
Fever
0
20
40
60
80
100
120
Percentage

TABLE 6

SIGNS OF CASES PRESENTING WITH LEPTOSPIROSIS n=156

Signs	No. of cases	Percentage
Jaundice	112	71.79
Hepatomegaly	51	32.69
Hypertension	49	31.41
Anemia	32	20.51
Splenomegaly	30	19.23
Hypotension	3	1.92
Polycythemia	1	0.64

Jaundice was found to be the predominant sign.

TABLE 7

MICROSCOPIC AGGLUTINATION TEST IN LEPTOSPIROSIS

No. of samples screened	No. of positive samples	Percentage
156	143	91.66

143 samples(91.66%) were positive for MAT

95% Confidence Interval is 86.51% - 95.3%.

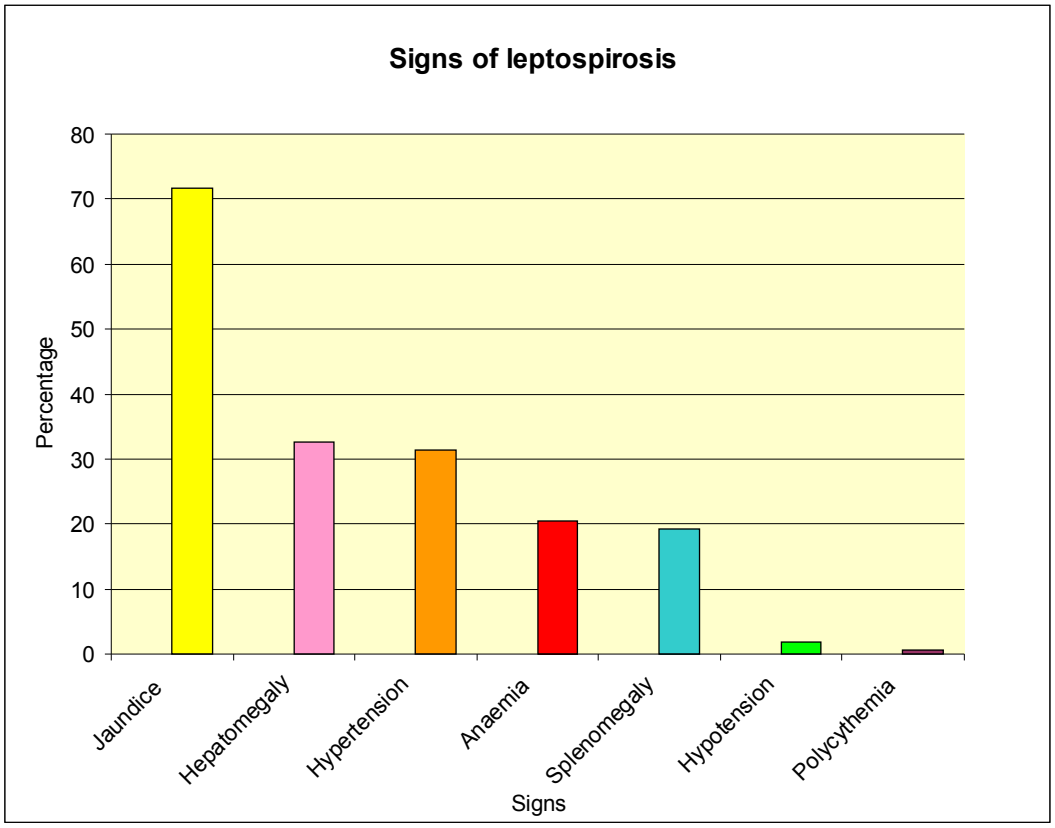

Signs of leptospirosis
Percentage
80
70
60
50
40
30
20
10
0
Jaundice
Hepatomegaly
Hypertension
Anaemia
Splenomegaly
Hypotension
Polycythemia
Signs

TABLE 8

SEROVAR DISTRIBUTION IN LEPTOSPIROSIS POSITIVE CASES n=143

Serovar	Positive titres			Total	Percentage
	1/80	1/160	1/320		
L.*icterohaemorrhagiae*	14	51	6	71	49.65
L.*australis*	10	25	6	41	28.67
L.*semaranga*	3	11	5	19	13.28
L. *grippotyphosa*	6	3	-	9	6.29
L.*canicola*	1	2	-	3	1.92
L.*louisiana*	-	-	-	-	-
L. *autumnalis*	-	-	-	-	-
L. *hebdomadis*	-	-	-	-	-

L. i*cterohaemorrhagiae* was the predominant serovar

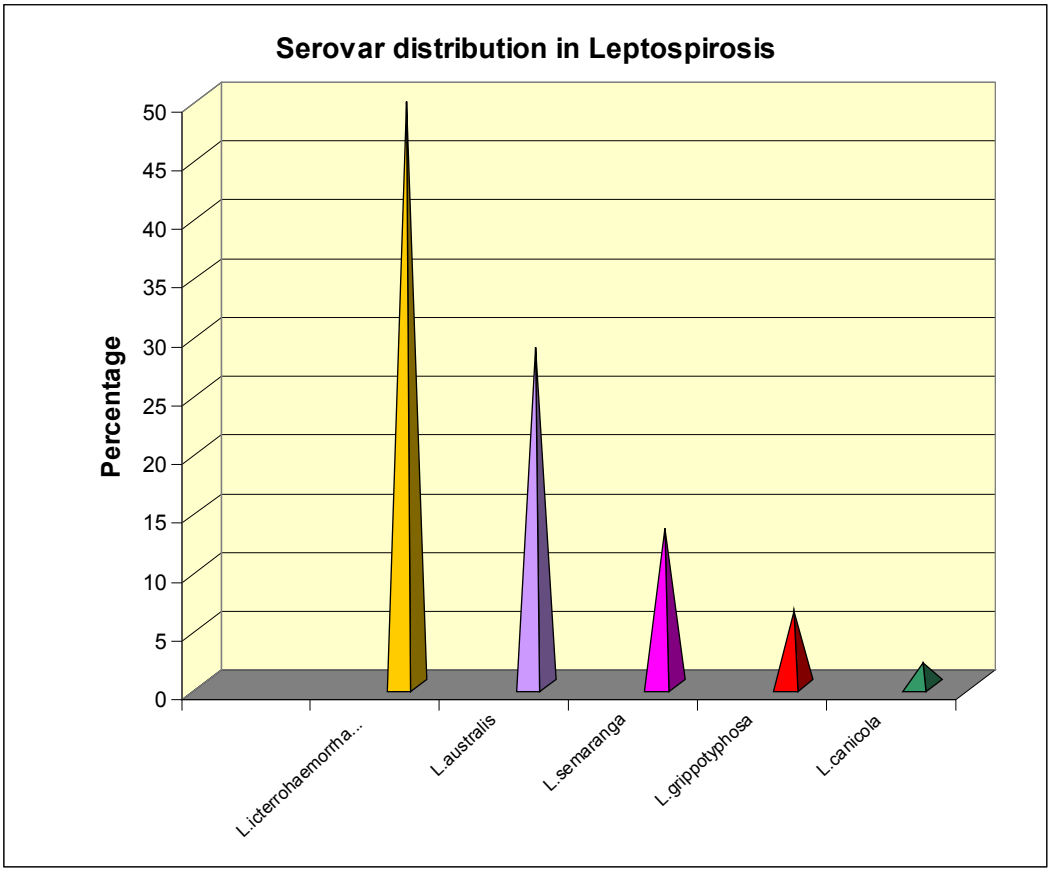
TABLE 9

SEROVAR DISTRIBUTION IN JAUNDICE CASES N=112

Serovar	No. of cases	Percentage
L. *icterohaemorrhagiae*	75	66.96
L. *australis*	24	21.42
L. *semaranga*	11	9.82
L. *grippotyphosa*	2	1.78
L. *canicola*	-	-
L. *louisiana*	-	-
L. *autumnalis*	-	-

L. *hebdomadis*	-	-

L. *icterrohaemorrhagiae* was the predominant serovar in jaundiced patients



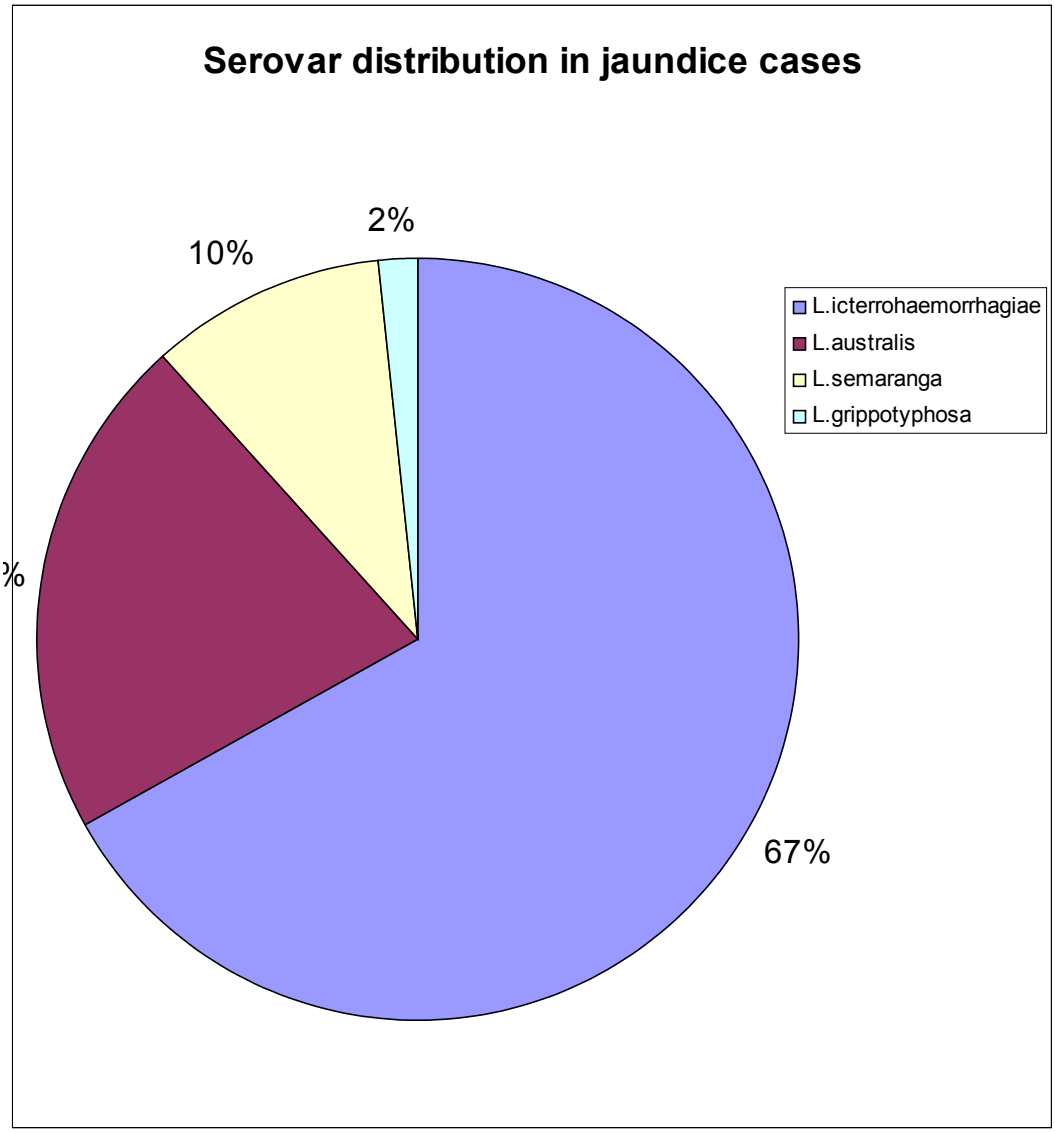

Serovar distribution in jaundice cases
2%
10%
%
67%
L.icterrohaemorrhagiae
L.australis
L.semaranga
L.grippotyphosa

TABLE 10

INVESTIGATIONS IN PATIENTS WITH LEPTOSPIROSIS N=156

Investigations	No. of cases	Percentage
Elevation of ESR	156	100
Abnormal LFT	112	71.79
Thrombocytopenia	79	50.64
Abnormal RFT	42	26.92
Abnormal LFT and RFT	34	21.79

ESR raised in all cases (100%), followed by abnormal LFT in 71.79% of patients

TABLE 11

TOTAL WBC COUNT IN LEPTOSPIROSIS n= 156

WBC count	No. of cases	Percentage
4,000-10,000	86	55.12
>10,000	44	28.20
<4,000	26	16.66

WBC count was normal in 55.12% of patients

TABLE 12

DIFFERENTIAL COUNT IN LEPTOSPIROSIS n= 156

Differential count	No. of cases	Percentage
Polymorphs increased	104	66.66
Lymphocytes increased	17	10.89
Both normal	35	22.34

Polymorphs % was increased in 66.66% of patients

TABLE 13

ELEVATION OF ESR IN LEPTOSPIROSIS n= 156

Level raised	No. of cases	Percentage
20-50mm/hr	118	75.64
>50mm/hr	38	24.35

ESR was moderately increased in 75.64% of patients

TABLE 14

PLATELET COUNT IN LEPTOSPIROSIS n=156

Platelet Count cells/cmm	No. of cases	Total cases	Percentage
1,50,000 – 2,50,000	77	77	49.35
1,00,000 – 1,50,000	39	79	50.64
50,000 – 1,00,000	31		
<50,000	9		

Thrombocytopenia was seen in 50.64% 0f patients.

TABLE 15

RENAL FUNCTION TESTS IN LEPTOSPIROSIS n=156

Renal parameters	Range	No. of cases	Total cases	Percentage
Creatinine mgm/dl	<1.5	114	114	73.07
	1.5 -2.0	26	42	26.92
	2.0 – 2.5	5		
	2.5 – 3.0	1		
	>3.0	10		
Urea mgm/dl	20 - 40	114	114	73.07
	40 – 50	15	42	26.92
	50 - 100	16		
	>100	11		

Blood urea, Serum creatinine raised in 26.92 % of patients.

TABLE 16

ACUTE RENAL FAILURE IN LEPTOSPIROSIS n=156

Total cases Screened	No. of cases	Percentage
156	2	1.28

2 patients(1.28%) went in for acute renal failure

TABLE 17

CORRELATION OF INCREASED RENAL PARAMETERS WITH PLATELET COUNT n=42

Parameters	Platelet count	No. of cases	Percentage
Increased Blood Urea and Serum Creatinine	< 1,50,000	38	90.47
	1,50,000 – 2,50,000	4	9.52

Renal parameters were found to be increased in more number of thrombocytopenic patients.

TABLE 18

SERUM BILIRUBIN (TOTAL) IN LEPTOSPIROSIS n=156

Serum Bilirubin mgm/dl	No. of cases	Total cases	Percentage
<1	44	44	28.20
1-5	76	112	71.79
5 - 10	15		
>10	21		

Total serum bilirubin raised in 71.79% and normal in 28.20%.

TABLE 19

SERUM GLUTAMIC OXALO ACETIC TRANSAMINASE (SGOT) IN LEPTOSPIROSIS N=156

SGOT IU/L	No. of cases	Total cases	Percentage
5-40	62	62	39.74
41 - 60	30	94	60.25
61 - 80	25		
81 - 100	14		
>100	25		

SGOT was found to be normal in 39.74%, and raised in 60.25%.

TABLE 20

SERUM GLUTAMIC PYRUVIC TRANSAMINASE (SGPT) IN LEPTOSPIROSIS n=156

SGPT IU/L	No. of cases	Total cases	Percentage
7-35	65	65	41.66
36 – 60	31	91	58.33
61 – 80	30		
81 - 100	9		
>100	21		

SGPT was found to be normal in 41.66% and raised 58.33%.

TABLE 21

SERUM ALKALINE PHOSPHATASE (SAP) IN LEPTOSPIROSIS n=156

SAP IU/L	No. of cases	Total cases	Percentage
15 - 121	66	66	42.30
I21 -150	10	90	57.69
151- 180	7		
>180	73		

SAP was found to be raised in 57.69%.

TABLE 22

RADIOLOGY IN LEPTOSPIROSIS n=156

Parameter	Findings	No. of cases	Percentage
X ray chest	Bilateral Lower Lobe Opacity	4	2.56%

Pulmonary involvement was present only in 4 (2.56%) patients

TABLE 23

CONCOMITANT INFECTION OF LEPTOSPIROSIS WITH DENGUE, HEPATITIS B n=156

Dual infections	No. of cases	Percentage
Leptospirosis with dengue	4	2.56%
Leptospirosis with hepatitis B	1	0.64%

Out of 156 samples ,four (2.56%) were positive for dengue IgM antibodies and one for HBsAg.

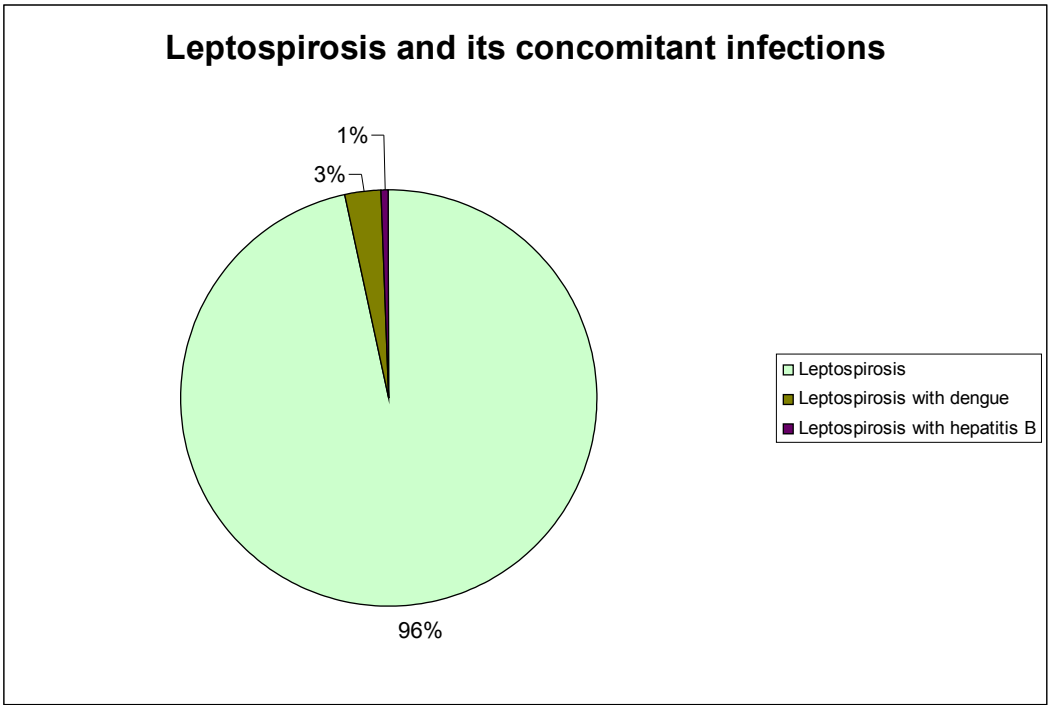

Leptospirosis and its concomitant infections
1%
3%
96%
Leptospirosis
Leptospirosis with dengue
Leptospirosis with hepatitis B

TABLE 24

CLINICAL SIGNS AND SYMPTOMS IN PATIENTS WITH DENGUE COINFECTION n=4

Signs and symptoms	No. of cases	Percentage
Fever	4	100
Myalgia	3	75.00
Hemorrhage	1	25.00
Hepatomegaly	1	25.00
Splenomegaly	1	25.00
Rash	-	-
Conj.suffusion	-	-
Abdominal pain	-	-
Jaundice	-	-

TABLE 25

LABORATORY PARAMETERS IN PATIENTS WITH DENGUE COINFECTION n=4

Parameters	No. of cases	Percentage
Differential count		
Polymorphs raised	2	50.00
Lymphocytes raised	2	50.00
PLATELET COUNT/cu.mm		
>2.5 lakhs	1	25.00
1.0 -1.5 lakhs	2	50.00
<50,000	1	25.00

TABLE 26

CLINICAL SIGNS AND SYMPTOMS IN PATIENT WITH HEPATITIS B n=1

Signs and symptoms	Duration
Fever	8 days
Headache	8 days
Myalgia	8 days
Vomiting	6 days
High coloured urine	4 days

TABLE 27

LABORATORY PARAMETERS IN PATIENT WITH HEPATITIS B COINFECTION n=1

Parameters		Values	Interpretation
Total WBC count/cu.mm		8,750	Normal
Differential count	Polymorphs	85%	Increased
	Lymphocytes	12%	Decreased
ESR mm/hr		29	Increased
Blood urea mgms/dl		56	Increased
Serum creatinine mgms/dl		3.4	Increased
Serum bilirubin-total mgms/dl		21.8	Increased
Serum bilirubin-direct mgms/dl		15.2	Increased
Serum bilirubin- indirect mgms/dl		6.6	Increased
SGOT IU/L		172	Increased
SGPT IU/L		78	Increased
SAP IU/L		190	Increased

Discussion

DISCUSSION

Leptospirosis is considered as an emerging disease and this has led to an increased interest and demand for information, notably in developing countries. This infection is a public health threat due to its morbidity and mortality. The detailed knowledge of its clinical features and other laboratory parameters is necessary for early clinical diagnosis which is then confirmed by serological tests.

The cross sectional study was conducted over a period of one year (March 2007-February 2008). Out of 378 samples which were with clinical features suggestive of leptospirosis, MSAT was positive in 156 samples (41.26%)(Table 1). MSAT is a rapid test used to identify if the person is infected or not. It detects the IgM antibodies. Sensitivity increases after a week's time. Galton et al found that patoc strain with killed Ag used for MSAT was more sensitive in the second week of illness.[26] The usefulness of MSAT as a screening test has been evaluated by Sumathi et al.[83] They have concluded that MSAT is a simple, quick and sensitive test which can be used as a screening test for laboratories in developing countries.

In this study the highest number of cases were in the 21 – 30 years age group (23.71%) followed by 31 - 40 years age group (19.87%) (Table 2). The pattern of age distribution might be due to increased outdoor activity among the 21 – 30 years age group which increases chance of exposure to infection. In the study by Margarita R et al, the mean age affected was 36 years.[50]

There is very slight increase in the incidence in males (51.28%) compared to females (48.71%) (Table 3) in this study. This correlates with the Pappachan et al study in which 58.9% were men.[59]

Higher incidence of leptospirosis occurs following rainfall.[76,77] In this study, leptospirosis cases occurred throughout the year although the number increased during the rainy season (Table 4). This may be due to the polluted environment which is an important epidemiological risk factor. This correlates with Sumathi G et al study (2004- 2006).[84] In Sharma KK et al study* the highest incidence of leptospirosis was during the rainy season.

In the present study the predominant symptoms were fever (100%), followed by high coloured urine (71.79%), vomiting (48.07%), headache (40.38%)(Table 5). The other symptoms were abdominal pain (34.61%), diarrhoea and cough (33.97%), myalgia (32.05%), breathlessness (19.87%), conjunctival suffusion (10.89%), hemoptysis (5.76%), dysuria (8.33)%, oliguria (5.12%), altered sensorium and haematuria (4.48%). In Sritharan M et al study all the patients had fever with chills and myalgia (100%).[81] In De A et al study fever was present in all cases (100%), myalgia in 51.35%, jaundice and conjunctival suffusion in 32.43% and altered sensorium in 13.51% of patients.[13]

In the study conducted by MA Muthusethupathy et al.(1995) fever, myalgia and jaundice formed 100% followed by renal involvement in 77.7%, conjunctival suffusion 72.7%, gastrointestinal symptoms 72%, meningeal signs 22.2%, splenomegaly 5.5% and haemorrhagic symptoms forming 3%.[55]

In the study done by Margarita R et al(1999) fever constituted 100%, followed by headache 50%, epigastric pain 42%, jaundice 73% conjunctival suffusion 61.5%.[50]

The most common signs observed in this study were jaundice (71.79%) and hepatomegaly (32.69%) (Table 6). This is in accordance with Margarita R et al study in which jaundice was present in 70.2% and hepatomegaly in 38.4% of patients.[50] In Gancheva G et al study (2005) hepatosplenomegaly 100%, jaundice 75%, hypotension 33.33%, and

haemorrhagic skin lesions 30.95% were the common signs.[27] In Marcial M R et al study jaundice 70.2%, oligoanuria 43.3%, hepatomegaly 18.3%, GI bleeding 17.3%,hematuria11.5% constitutes the predominant signs.[49]

The differences in distribution of major clinical features in this study might be due to the fact that the signs and symptoms keep varying from time to time as the infecting serovar also keeps changing with time

In the present study out of the 156 samples which were positive by MSAT, 143 (91.66%) turned positive for MAT which is the Gold standard method (Table 7). The predominant serovar were L.*icterrohaemorrhagiae* (49.65%) followed by L.*australis* (28.67%), L.*semaranga* (13.28%), L.*grippotyphosa* (6.29%) and L.*canicola* (1.92%) (Table 8). Along with these serovars L.*louisiana*, L.*autumnalis*, and L.*hebdomadis* were included in the panel but these serovars were not causing leptospirosis in this study. In Sumathi G et al(2004 – 2006) study L.*icterrohaemorrhagiae* (48.0%) was the predominant serovar followed by L.*australis* (37.0%) and L.grippotyphosa (26.0%).[84] In the study by Sritharan et al in Hyderabad (2007), L.*icterrohaemorrhagiae* was the predominant serovar (68%) followed by L.*australis* 22%, L.*autumnalis* 8%, L.javanica 2%.[81] In 1995 the study conducted by Leptospirosis Research Cell (LRC) revealed L.*autumnali*s as the common serovar[84.] Now the present study revealed L.*icterrohaemorrhagiae* as the predominant serovar. This indicates the seroprevalence of leptospirosis in different periods have shown various serogroups to be predominant.

In jaundice cases L.*icterrohaemorrhagiae* was found to be the predominant serovar . (66.96%), followed by L.*australis* 21.42%, L.semaranga 9.82%. L.*grippotyphosa* 1.78% (Table 9) in this study. In Debnath C et al (2004) study in jaundice cases L.*pomona* (80%) and

L.*grippotyphosa* (20%) were the predominant serovar and none of the patients sera reacted against L. *icterrohaemorrhagiae*.[14] The seroprevalence of the serovars in jaundiced leptospirosis patients was also studied by Joseph et al (1966) in Northern India. They found the prevalence of L.*icterrohaemorrhagiae* as 69.76%.[36]

Other laboratory parameters were done for all patients in which ESR was raised in all cases (100%), followed by abnormal LFT in 71.79%, thrombocytopenia in 50.64%, abnormal RFT in 26.92% and both abnormal LFT and RFT in 21.79% of patients (Table 10)..In Sritharan M et al study abnormal RFT was seen in 52% and abnormal LFT in 44%of patients.[81] In Gancheva et al study (2005) ESR was elevated in 86.90%, thrombocytopenia 42.86%, abnormal RFT in 72.62%, abnormal LFT in 70.24%.[27]

White Blood Cell count was normal in 55.12% ,increased in 28.20%, decreased in 16.66% of patients in the present study (Table11). This is in contrary to Marcial M.R. et al study in which White Blood Cell count was normal in 30.8% and leucocytosis in 69.2%.[49] In Gancheva G et al*(2007) study leucocytosis was seen in 61.29%.[28]

Differential Count shows percentage of polymorphs were increased in 66.66% (Table 12) of patients in the present study. In Gancheva G et al study polymorphs % were increased in 95.12%.[28]

ESR was moderately raised (less than 50mm/hr) in 75.64% and extremely raised (more than 50mm/hr) in 24.35% (Table 13) in this study. This is in accordance with Gancheva G et al* (2007) study in which moderate rise in ESR was present in 74.39% and extreme rise in 20.73%.[28]

In this study platelet count was decreased in 50.64% of patients (Table 14).This

correlates well with the Gancheva G et al (2007) study which shows thrombocytopenia in 58.62%[28] and with Ramon Peces study in which thrombocytopenia was seen in 58% of patients.[69]

Blood urea, serum creatinine were increased in 26.92% of cases (Table 15) in this study. This is in contrary to Marcial M R et al study in which blood urea and serum creatinine were increased in 73.1% of patients[49] and with Margarita R et al study in which blood urea and serum creatinine were increased in 92.3% of patients.[50]

Only two cases (1.28%) out of 156 went in for Acute Renal Failure (Table 16). Both the cases after appropriate treatment recovered. No mortality was observed in this study. In Margarita R et al study, Acute renal failure was seen in 30.7%of patients.[50] In Marcial M R et al study Acute Renal Failure was seen in 74%[49] and in De A et al study it was seen in 16.2% of patients.[13] In Muthusethupathi MA et al (1994) study in Madras city during 1987-91 there were120[54] cases of Acute Renal Failure due to leptospirosis, but in the last two years there were only 15 cases of leptospiral ARF.[53] In the present study there were only 2 cases.This indicates a decline in the severity of the disease and now more number of milder form of disease occurs.

Blood urea, serum creatinine were increased in most patients of decreased platelet count (90.47%) than in patients with normal platelet count (9.52%) (Table 17). This correlates well with Ramon Peces study in which it was concluded that there exists a significant inverse correlation between platelet count and the renal parameters.[69]

There was an increase in SerumTotal bilirubin levels in 71.79% of patients (Table 18). This is in accordance with the Gancheva G et al (2007) study in which serum total bilirubin

was increased in 75% of patients[28] .and with Margarita R et al study in which it was increased in 73.6% of patients.[50]

Serum total bilirubin level is usually <20mgm/dl in leptospirosis.[70] This fact correlates with the present study in which serum Total bilirubin levels were less than 20mgm/dl in all the cases of leptospirosis except in a patient with Hepatitis B coinfection. The serum total bilirubin level in this patient was 21.8mgm/dl.

Serum Glutamic Oxalo Acetic Transaminase (SGOT) were normal in 39.74% and increased in 60.24% of patients (Table 19) in this study. In Gancheva G et al (2007) study SGOT was normal in 23.17% and increased in 76.83%.[28] In Margarita et al study SGOT was elevated in 94% of patients.[50]

Serum Glutamic Pyruvic Transaminase (SGPT) were normal in 36.53% and increased in 63.47% of patients (Table 20). In Gancheva G et al (2007) study 24.72% of patients showed normal and 75.28% increased SGPT.[28] In Margarita et al study SGPT was elevated in 94% of patients.[50]

In leptospirosis serum transaminases rarely exceed 200 IU/L* in contrast to acute viral hepatitis[66]. This fact correlates with the present study in which SGOT and SGPT were less than 200 IU/L in all the patients.

Serum Alkaline Phosphatase (SAP) was normal in 42.30% and increased in 57.7% (Table 21). Gancheva G et al study showed normal SAP in 23.53% and increased SAP in 76.47%.[28] In Margarita et al study SAP was elevated in 61.5% of patients.[50]

In radiological parameter, X ray chest showed bilateral lower lobe opacity in 4 cases

(2.56%) (Table 22). This indicates pulmonary involvement is rare in Chennai. In De A et al study pulmonary involvement was seen in 37.83%[13] and in Perani V et al study it was seen in 5.68%.[64]

Dual infection of leptospirosis with other infections have been reported.[1,39,40,] Leptospiral illness may be a significant component in cases of dual infections or in simultaneous infections with more than two pathogens.[3]

In the present study dual infection with dengue was seen in 4 cases (2.56%) (Table 23) as compared to 8% in Levett P. N. et al study in Barbados.[47] All the four patients were positive for dengue IgM antibodies and had Acute primary dengue infection. Out of the 4 patients , 2 were males and 2 were females, all in the age group of 20 – 30years. Fever (100%) and myalgia (75%) were the predominant symptom in these patients. Splenomegaly in one patient (25%), hepatomegaly in one patient (25%) and haemorrhage in one patient (25%) were the signs observed in these patients (Table 24).. The characteristic dengue rash was absent in all these patients which is contrary to Levett P N et al study in Barbados in which all cases had dengue rash.[47]

In this study the differential count in dengue dual infection patients showed increased polymorphs percentage in 2 patients (50%) and increased lymphocytes percentage in 2 patients (50%). Platelet count was normal in one patient (25%) and decreased in 3 patients (75%). Out of these three cases with decreased platelets, one had platelet count less than 50,000 (Table 25), which indicates poor prognosis. With fluid resuscitation, platelet transfusion, appropriate drug and supportive therapy, patients recovered. No mortality was observed in these patients.

Concomitant infection with Hepatitis B was seen in one patient (0.64%) (Table 23). No

Indian studies have been reported so far of leptospirosis with concomitant Hepatitis B infection. In Singapore, Kaushik et al reported one case of Weil syndrome and concomitant Hepatitis B infection.[40] In this study the patient was 30years old male,with symptoms of fever, headache, myalgia, vomiting and high coloured urine(Table 26). Laboratory parameters showed normal WBC count, polymorphs percentage increased, moderately raised ESR, renal parameters blood urea and serum creatinine increased. Serum total bilirubin, SGOT, SGPT, SAP were raised in this patient (Table 27).

In Kaushik et al study the patient was 30 years old male with complaints of epigastric pain, fever, chills and a reduced urine output. The signs observed were Jaundice, conjunctival suffusion, left conjunctival haemorrhage and flapping tremor. The laboratory parameters in the patient showed thrombocytopenia, ,increased blood urea and serum creatinine and increased serum total bilirubin with elevated SGOT, SGPT, and SAP.[40]

In the present study the patient with Hepatitis B concomitant infection recovered fully from leptospirosis, despite the combined hepatic involvement by these two illnesses and with the severe systemic manifestations of leptospirosis,

All the patients in the present study recovered with appropriate drug and supportive therapy. There was no mortality observed in this study . .

Summary

SUMMARY

- The number of leptospirosis cases in this study was 156(41.26%).
- Incidence was higher in 21 to 30 years age group (23.71%)
- There was very slight preponderance in males (51.28%).
- Leptospirosis occured throughout the year although the number of cases increased after rainy season.
- The most common symptoms observed were fever(100%), high coloured urine(62.82%),vomiting (48.07%) and headache(40.38%).
- Jaundice was seen in 71.79% of patients.
- Out of the 156 samples positive by MSAT, 143 (91.66%) were positive for Microscopic Agglutination Test (MAT).
- The common serovars were L. *icterohaemorrhagiae*(49.65%), followed by L. *australis*(28.64%), L. *semaranga(*13.28%), L.*grippotyphosa*(6.29%), and L. *canicola(*1.92%).
- L. *icterohaemorrhagiae* was found to be the predominant serovar in jaundice patients(66.96%).
- Elevated ESR was seen in all cases of leptospirosis, abnormal Liver Function Tests in 71.79%, Thrombocytopenia in 50.64%, abnormal Renal Function Tests in 26.92%,both abnormal RFT and LFT in 21.79% of patients were observed.
- White Blood Cell count was normal in 55.12% of patients, elevated in 28.20% and decreased in16.66% of patients.
- Differential Count showed polymorphs percentage were increased in 66.66% of patients.
- Moderate rise in ESR was seen in 75.64% of and extremely rised ESR in 24.35% of

patients

- Blood urea, serum creatinine were raised in 26.92% of patients.
- 2 cases(1.28%) out of 156 went in for Acute Renal Failure.
- Blood urea, serum creatinine were increased in more number of thrombocytopenic patients(90.47%) compared to non-thrombocytopenic patients(9.52%).
- Serum total bilirubin was normal in 28.20% and elevated in 71.79% of patients.
- Serum total bilirubin was less than 20mgm/dl in all cases of leptospirosis except in a patient with the Hepatitis B concomitant infection. The serum total bilirubin in this patient was 21.8mgm/dl.
- Serum Glutamic Oxalo Acetic transaminase was normal in 39.74% and elevated in 60.25% of patients..
- Serum Glutamic Pyruvic transaminase was normal in 41.66% and elevated in 58.33% of patients..
- Serum Alkaline Phosphatase was normal in 42.30% and elevated in 57.69% of patients..
- Pulmonary involvement was seen in 4 cases (2.56%).
- Concomitant infection of leptospirosis with dengue were seen in 4 cases (2.56%) and with Hepatitis B in 1 case (0.64%).
- All the 4 cases of concomitant dengue infection had primary dengue.
- Fever (100%), and Myalgia (75%) were the predominant symptom in dengue concomitant infection patients.
- Thrombocytopenia was seen in 3 cases (75%) of dengue dual infection patient.
- Fever, headache, myalgia, vomiting, high coloured urine were the symptoms presented by the Hepatitis B concomitant infection patient.
- Normal total WBC count, increased polymorphs%, moderately elevated ESR, increased

renal parameters, elevated serum total bilirubin with raised SGOT,SGPT and SAP were observed in Hepatitis B dual infection patient.

- All the patients recovered with appropriate drug and supportive therapy. No mortality was observed in this study.

Conclusion

CONCLUSION

- In conclusion, by correlating the biochemical and haematological parameters a quick clinical suspicion of leptospirosis can be made out and diagnosed early, for initiating appropriate treatment.
- The predominant serovar was L.*icterrohaemorrhagiae* (49.65%).
- Concomitant infection of leptospirosis with dengue and hepatitis B were seen and leptospirosis should not be missed in such scenario since antimicrobial agents for leptospirosis can reduce its severity and duration.
- There was no mortality observed in this study due to early recognition and intensive treatment, even in the severe form of disease.
- Early recognition and appropriate treatment leads to a favourable outcome of this potentially life threatening condition.

Annexure

PROFORMA

NAME: AGE: SEX: OCCUPATION:

IP No: ADDRESS: DATE:

PRESENTING SYMPTOMS WITH DURATION:

PRESENTING SIGNS:

H/O PRESENT ILLNESS:

TREATMENT GIVEN:

INVESTIGATIONS:

1. Haematological parameters: Hb%
   TC
   DC
   ESR
   Platelet Count
2. Biochemical parameters: LFT
   RFT
3. Radiological parameters: Chest X- ray
   Ultrasound Abdomen.

Abbreviations

ABBREVIATIONS

DNA	Deoxyribo Nucleic Acid
RNA	Ribo Nucleic Acid
CSF	Cerebro Spinal Fluid
MSAT	Macroscopic Slide Agglutination Test
MAT	Microscopic Agglutination Test
ELISA	Enzyme Linked Immunosorbent Assay.
HBsAg	Hepatitis B Surface Antigen.
EMJH	Ellinghausen – McCullough- Johnson-Harris
LFT	Liver Function Tests
RFT	Renal Function Tests
WBC	White Blood Cells
ESR	Erythrocyte Sedimentation Rate
SGOT	Serum Glutamic Oxalo Acetic Transaminase
SGPT	Serum Glutamic Pyruvic Transaminase
SAP	Serum Alkaline Phosphatase

Appendices

APPENDIX – I

EMJH MEDIUM:

(Ellinughausen – Mccullough – Johnson – Harris medium) composition of EMJH medium base (Johnson Harris 1967).

.**EMJH BASE-(DIFCO LABORATORIES, USA)**

Ingredients	**gms/lit**
Sodium phosphate diphasic	1
Potassium	3
Sodium chloride	1
Ammonium chloride	0.25
Thiamine	0.005

EMJH ENRICHMENT:

The enrichment bovine serum albumin (BSA) at the proportion of 1gm of BSA per 10 ml of EMJH medium at a final concentration of 10%. It was then, filtered through a membrane (pore size .22µ) and was added as enrichment after cooling.

.

EMJH LIQUID MEDIUM:

Liquid EMJH medium was prepared by dissolving 2.3 gms of EMJH base in 900 ml of triple distilled water, the pH is adjusted to 7.5. After autoclaving at 15 lbs for 15 minutes the medium was added aseptically with enrichment at 10% level. 100ml of enrichment is added to 900ml of EMJH medium. Later it was distributed aseptically in screw capped tubes. It was checked for sterility after 24 hrs at 27ºC

.**EMJH SEMISOLID MEDIUM:**

To prepare EMJH semisolid medium 0.2% bacteriological agar was added to the EMJH base and the complete medium was prepared as above.

SELECTIVE AGENT:

5-Fluoro uracil was used as selective agent. Leptospira are resistant to the antibacterial activity of pyrimidine analogue,5-Fluora uracil.

100ml of 5FU was added to 5ml of distilled water. To this 0.1 to 0.2 ml of 0.1N NaOH was added and heated gently to dissolve 5-FU completely, The pH was adjusted t0 7.4 – 7.6 and the volume was made upto 10ml with distilled water. The solution was sterilised by filtration through membrane filter(pore size 0.45µm). Add 1 ml of this into 100ml of EMJH medium to get the final concentration of 100 µgm per ml.

CULTURING OF LEPTOSPIRA:

Appropriate amount of organism was transferred to EMJH liquid and semisolid media. A period of 7 days incubation at room temperature (25-31ºC) was maintained. The culture was then examined under DFM to check for the required growth and active motility of leptospira. Growth was also visualized as one or more rings, several mm below the surface of the semisolid medium. Once growth was found optimal, it was used as live antigen.

APPENDIX – II

REAGENTS:

1. **Phosphate Buffered Saline (PBS):**

Ingredients	**:**	**g/l**
Sodium chloride	:	8.0
Potassium chloride	:	0.2
Disodium hydrogen phosphate	:	1.15
Potassium dihydrogen phosphate	:	0.2
Distilled water	:	1000 ml

pH was adjusted to 7.4.

The above ingredients were dissolved in sterile distilled water, and then filtered using filter paper

APPENDIX – III

DENGUE IgM CAPTURE ELISA : (PANBIO)

PRINCIPLE:

- Serum antibodies of IgM class when present combine with antihuman IgM antibodies attached to the polystyrene surface of the microwell test strips (assay plate).
- A concentrated pool of dengue 1 to 4 antigens is diluted to the correct working volume with antigen diluent.
- An equal volume of HRP conjugated monoclonal antibody (MAb) is added to the diluted antigen, which allows the formation of antigen-MAb complexes.
- Residual serum is removed from the assay plate by washing and complexed antigen-MAb is added to the assay plate.
- After incubation, the microwells are washed and a colourless substrate system tetramethylbenzidine/Hydrogen peroxide (TMB/H_2O_2) is added.
- The substrate is hydrolysed by the enzyme and the chromagen changes to a blue colour.
- After stopping the reaction with acid, The TMB becomes yellow.
- Colour development is indicative of the presence of anti dengue IgM antibodies in the test sample.

KIT COMPONENTS:

- Anti-human IgM coated microwells
- Dengue 1-4 antigens (Recombinant)
- Wash buffer concentrate
- Serum diluent
- Antigen diluent
- HRP conjugated monoclonal antibody tracer
- Tetramethyl benzidine TMB
- IgM positive control serum
- IgM cut off calibrator serum
- IgM negative control serum
- Stop solution

HBsAg ELISA: (HEPALISA)

KIT COMPONENTS:

- Microwells coated with anti- HBsAg (monoclonal)
- Enzyme Conjugate Concentrate.
- Conjugate Diluent.
- Wash Buffer Concentrate.
- TMB Concentrate.
- Substrate (TMB Diluent).
- Negative control serum
- Positive control serum.
- Stop solution.

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INSTITUTIONAL ETHICAL COMMITTEE
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K.Dis.No.16328 P & D3/Ethies/Dean/GGH/08 Dated: 08.09.2008

Title of the work : CORRELATION OF DIFFERENT LABORATORY PARAMETERS IN THE DIAGNOSIS OF LEPTOSPIROSIS

Principal Investigator : Dr. B. Ananthi

Department : Institute of Microbiology, MMC, Chennai – 3.

The request for an approval from the Institutional Ethical Committee (IEC) was considered on the IEC meeting held on 10th Sept. 2008 at 2 p.m. in GGH, Dena's Chamber, Chennai - 3

The members of the Committee, the Secretary and the Chairman are pleased to approve the proposed work mentioned above, submitted by the principal investigator.

The principal investigator and their term are directed to adhere the guidelines given below:

1. You should get detailed informed consent from the patients/participants and maintain confidentiality.
2. You should carry out the work without detrimental to regular activities as well as without extra expenditure to the Institution or Government.
3. You should inform the IEC in case of any change of study procedure, site and investigation or guide.
4. You should not deviate form the area of the work for which I applied for ethical clearance.
5. You should inform the IEC immediately in case of any adverse events or serious adverse reactions.
6. You should abide to the rules and regulations of the institution(s)
7. You should complete the work within the specific period and if any extension of time is required, you should apply for permission again and do the work.
8. You should submit the summary of the work to the ethical committee on completion of the work.
9. You should not claim funds from the Institution while doing the work or on completion.
10. You should understand that the members of IEC have the right to monitor the work with prior intimation.

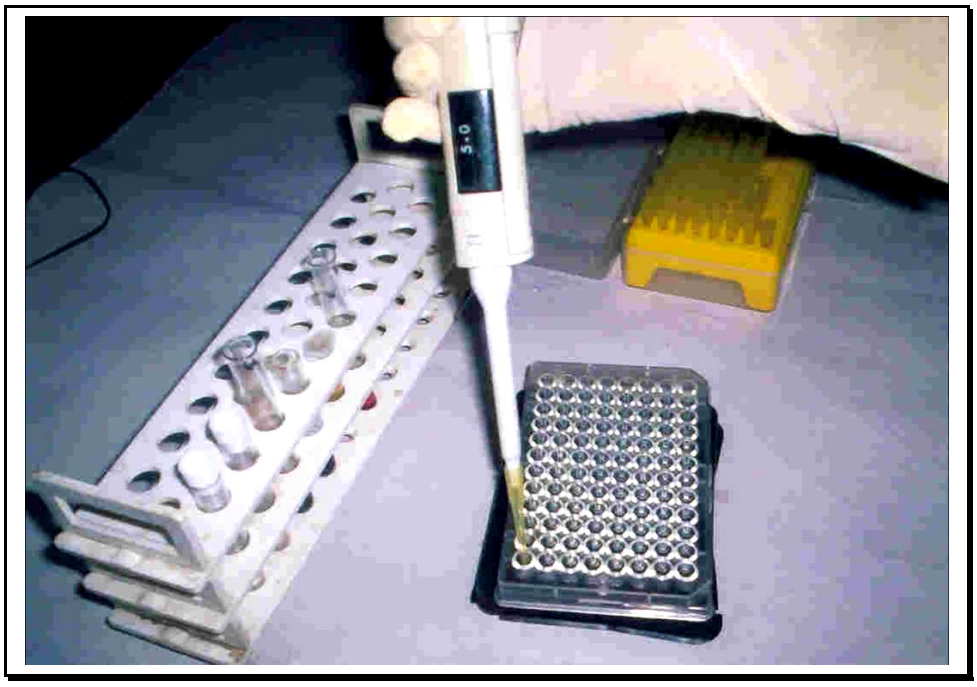
SECRETARY
IEC, GGH,CHENNAI

CHAIRMAN
IEC, GGH,CHENNAI

DEAN
GGH &MMC, CHENNAI

RKM.5.6(2)

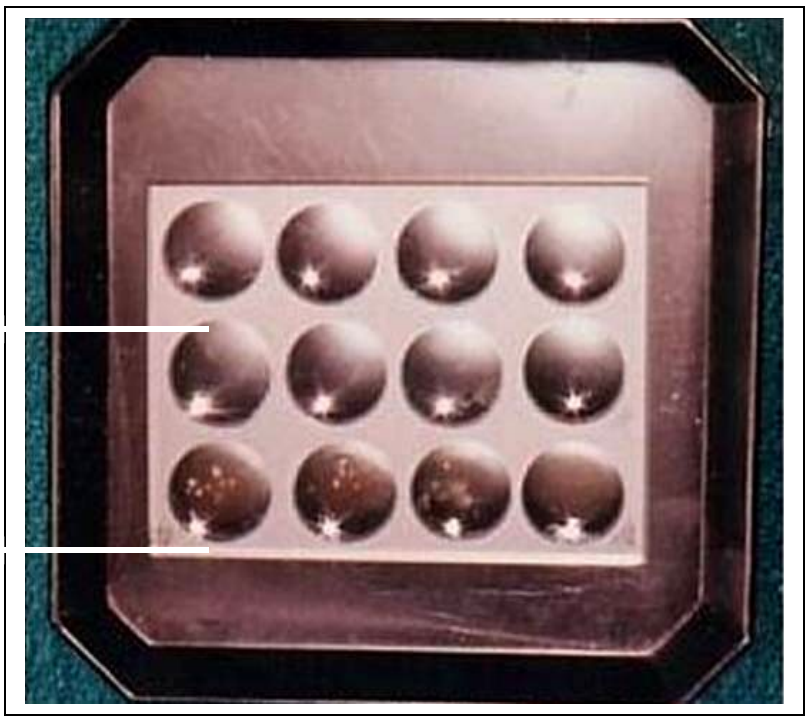
MSAT SETUP



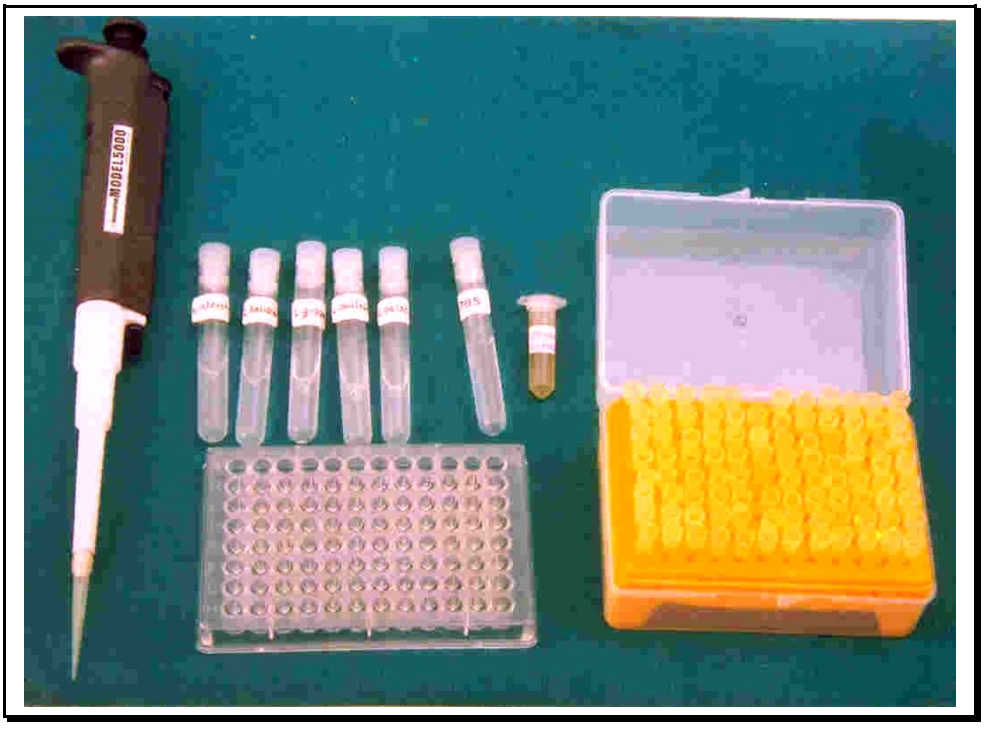
MSAT (POSITIVE & NEGATIVE)

Negative

Positive



MAT SETUP



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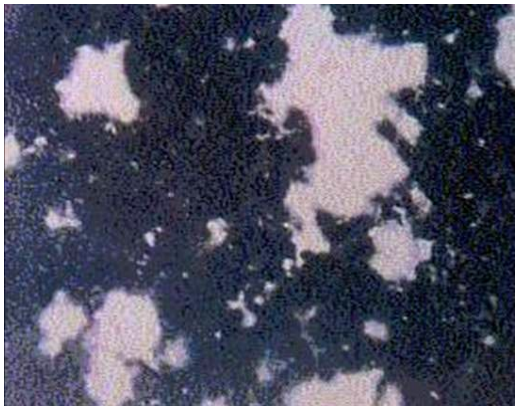

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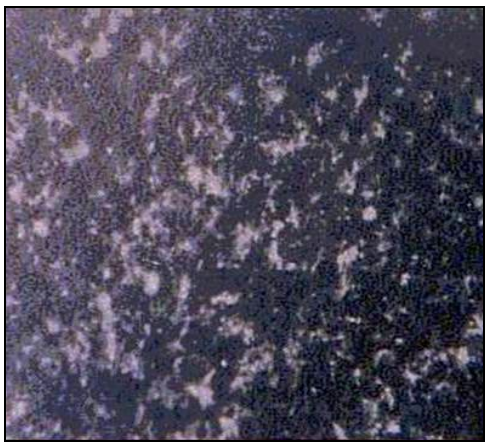
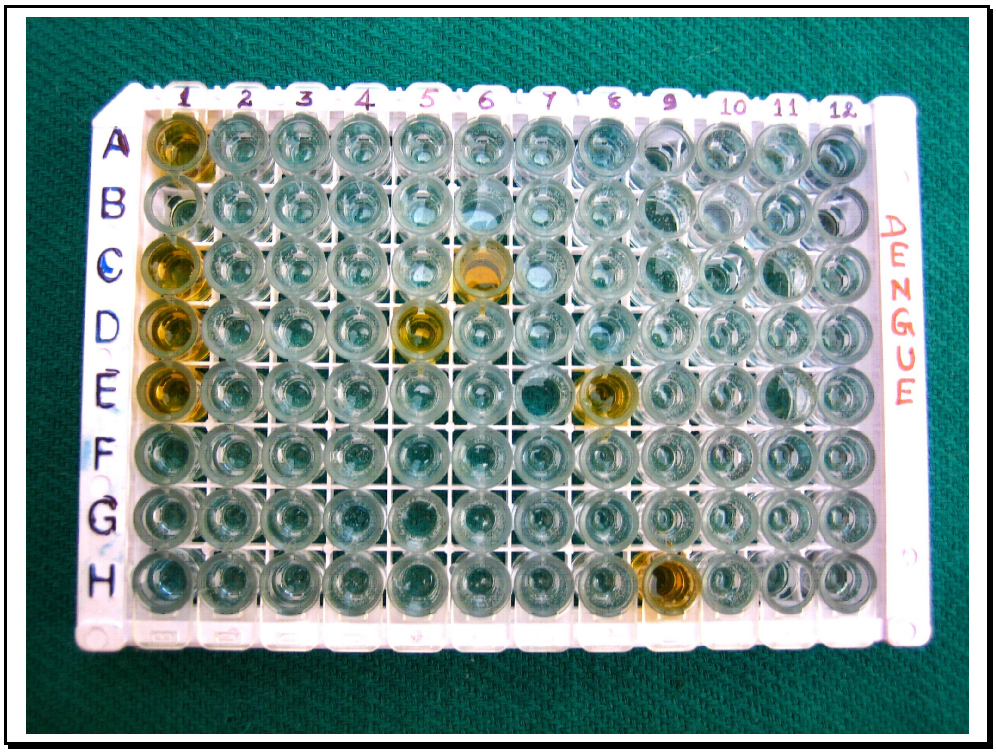
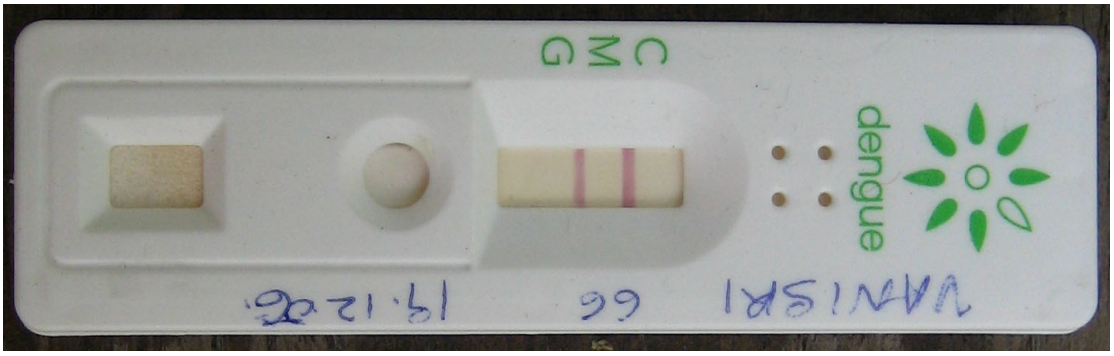


DENGUE IGM CAPTURE ELISA - TEST RESULT



A_1-Positive control, B_1-Negative control, C_1,D_1 and E_1-cut off calibrator control

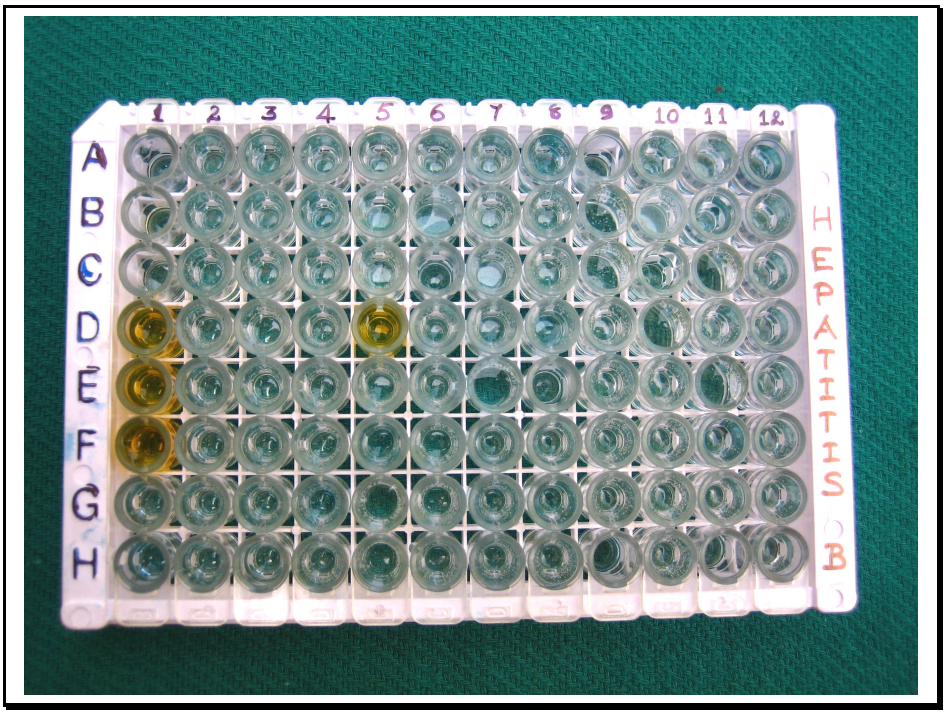
RAPID TEST KIT FOR DENGUE



Positive for Dengue IgM - Anti bodies

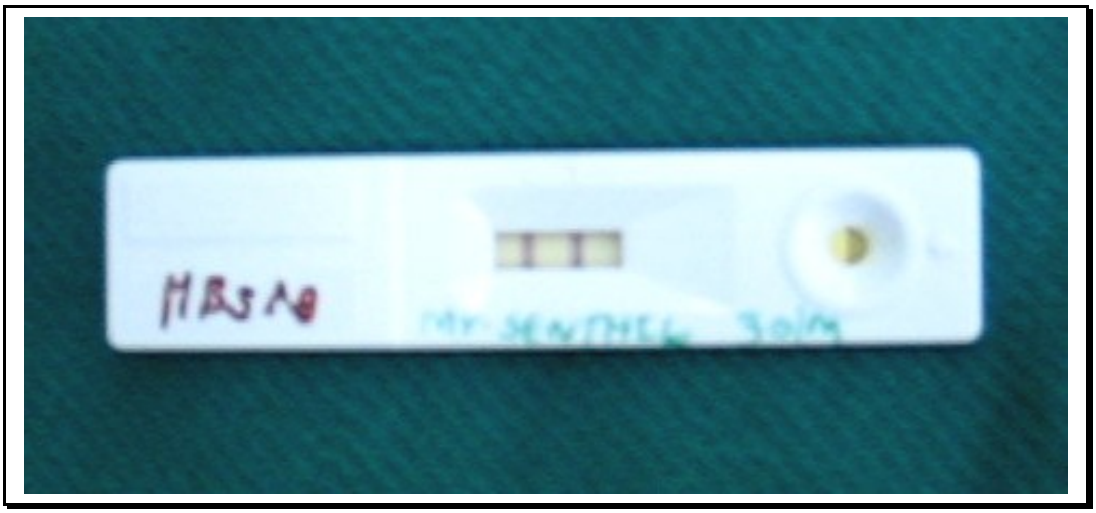
C-Control, M- IgM Antibodies, G-IgG Antiboides

HBS AG CAPTURE ELISA - TEST RESULTS



A_1-Blank, B_1 and C_1-Negative Control, D_1,E_1 and F_1-Positive Control

RAPID TEST KIT FOR HEPATITIS B



Positive for HBs Ag.