**A Dissertation on**

# PREVALENCE AND DETECTION OF LEPTOSPIROSIS AMONG VOLUNTARY BLOOD DONORS

*Dissertation submitted to*

**THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY**

*In partial fulfillment of the regulations*

*for the award of the degree of*

## M.D. BRANCH - XXI

**IMMUNOHAEMATOLOGY &**
**BLOOD TRANSFUSION**


**DEPARTMENT OF TRANSFUSION MEDICINE**

**THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY**

**CHENNAI, INDIA**

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# CONTENTS

## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| CMV | - | Cytomegalo Virus |
| DFM | - | Dark field Microscopy |
| EBV | - | Ebstein – Barr Virus |
| HAV | - | Hepatitis `A' Virus |
| HBsAg | - | Hepatitis `B' surface antigen |
| HCV | - | Hepatitis `C' Virus |
| HEV | - | Hepatitis `E' Virus |
| HGV | - | Hepatitis `G' Virus |
| HHV – 6 | - | Human herpes virus 6 |
| HHV-8 | - | Human herpes Virus – 8 |
| HIV | - | Human Immunodeficiency Viruses |
| HPV-B19 | - | Human Parvo Virus B19 |
| HTLV | - | Human T-cell lymphotropic Virus |
| MAT | - | Microscopic Agglutination Test |
| PBS | - | Phosphate buffered saline |
| PCR | - | Polymerase Chain Reaction |
| SAT | - | Slide Agglutination Test |
| TBE | - | Tris Borate EDTA buffer |
| TTD | - | Transfusion Transmitted Diseases |
| TTV | - | Transfusion Transmitted Virus |
| VDRL | - | Venereal Disease Laboratory Testing |
| WNV | - | West Nile Virus |

# APPENDIX

# MAINTENANCE OF LEPTOSPIRES

## ROUTINE CULTURES

Stains used routinely as sources of antigens for serological tests or other purposes are maintained in liquid medium dispensed in 5 ml amounts in screw capped test tubes. Duplicate tubes should be inoculated. The inoculum should be approximately 10% of culture volume and should be examined microscopically to confirm the presence of viable organisms and the absence of contamination, culture are kept at room temperature after incubatio of 5-7 days at 30°C. Cultures are routinely transferred at 2-3 weekly intervals.

## STOCK CULTURE

Stock Culture are best maintained in tubes of semi-solid media and stored in dark at room temperature. Cultures are liable for at least 3 months and upto 1 year.

# The Tamilnadu Dr. M.G.R. Medical University

## Department of Transfusion Medicine

Licence No. 191/28C

## BLOOD DONOR FORM

THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY

| Blood Bag No. | Date | | | Group & Rh |
|---|---|---|---|---|
| | | | | |

### Personal Particulars

| Donor's Name | Age : | Sex : Male / Female |
|---|---|---|
| | | |

| Residence Address | Office Address |
|---|---|
| ..... | ..... |
| ..... | ..... |
| ..... Ph : ..... | ..... Ph : ..... |

## KIND ATTENTION

Kindly furnish the following information sought on medical grounds as per Government Notification. If any question is felt embarrassing kindly bear with us

## TEMPORARY DEFERRAL, IN THE PAST 12 MONTHS HAVE YOU

- Received Tranfusion of Blood or its products Y/N
- Suffered from Hepatitis or had Hepatitis Immunoglobulin or had close contact with an individual suffering from Hepatitis Y/N
- Had exposure to tattoos, acupunture or body piercing? Y/N
- Had antirabies vaccine or was treated for dog bite? Y/N
- Undergone any major surgery or met with any major acciddent? Y/N

## IN THE PAST 6 MONTHS HAVE YOU EVER

- Suffered from Typhoid / Cholera / Acute infection of kidney or Bladder Y/N
- Had delivery / had pregnancy / any abortion / or been breast feeding? Y/N / N/A*
- Had any major surgery or met with any minor accident? Y/N

* N/A - Not applicable

## IN THE PAST 3 MONTHS

- Have you donated blood, plasma or platelets? Y/N
- Have yow been treated for malaria? Y/N
- Have you had any history of measles, mumps and chickenpox? Y/N

## IN THE PAST 1 MONTH

- Had treatment for acne with Isotretinoin? Y/N
- Had Anti tetanus serum, Anti venom serum, Anti diphtheria serum, Anti gas gangrene serum or Rubella vaccination? Y/N

## IN THE PAST 3 WEEKS

- Have you had tooth extraction or any dental procedure? Y/N

## IN THE PAST 2 WEEKS

- Have you had chicken pox, shingles, measles, mumps or yellow fever vaccination Y/N

## IN THE PAST 1 WEEK

- Have you had cortisone for treatment? Y/N
- Had history of diarrhea with fever? Y/N

## IN THE PAST 4 DAYS

- Have you had IV antibiotics? Y/N

## IN THE PAST 3 DAYS

- Have you had oral antibiotics? Y/N

## IN THE PAST 24 HOURS

- Have you had alcoholic drinks Y/N
- Are you an aircrew, a heavy machine vehicle driver, a construciton worker? Y/N
- Are you reporting for duty in the next 12 hours? Y/N
- Are you suffering from cold, cough, sore throat or acute sinusitis? Y/N

## PERMANENT DEFERRAL

H/o. Uncontrolled blood pressure or stroke? Y/N

H/o. Heart disease or arrhythmias? Y/N

H/o. Epilepsy or anticonvulsants? Y/N

H/o. Auto immune disease or immounsuppressive therapy? Y/N

H/o. Abnormal bleeding tendencies? Y/N

H/o. Diabetic mellitus on treatment with insulin or hypoglycemic drugs? Y/N

H/o. Chronic liver disease or endocrine disorders? Y/N

H/o. Diabetic mellitus on treatment with insulin or hypoglycemic drugs? Y/N

H/o. Chronic liver disease or endocrine disorders? Y/N

H/o. Parkinsons diseases? Y/N

H/o. Psorasis or treatment for the same? Y/N

H/o. Psychiatric disorders? Y/N

H/o. Major surgeries for kidney, heart, liver or brain? Y/N

H/o. Severe allergic disorders or asthmatic on steroid therapy? Y/N

H/o. IV drug abuse, heterosexual/homosexual promiscuity / STD? Y/N

## GENERAL QUESTIONS

1. Have you donated blood? Y/N
2. When was your last blood donation? .....

   How many times have you donated? .....
3. Are you willing to donate for emergency situations? Y/N
4. Have you had any reactions like giddiness/fainting attacks/ fits after donation? Y/N
5. Any history of unexplained weight loss/ chronic cough / fever / diarrhoea / Lymph nodes enlargement? Y/N

## DECLARATION

I hereby declare that the above information is true to the best of my knowledge and this consent of mine to be a blood donor is voluntary. I understood that certain tests (HIV, HCV, HBV, SYPHILIS, MALARIA), will be performed on my blood for the purpose of ensuring the safety.

I would like to know the results, if any positive. Y/N

Date Signature of donor

## PHYSICAL EXAMINATION

| Wt (in Kg) | HB gms % | PR | BP | RR | TEMP. | CVS | RS | CNS | ABD | Skin disease at phlebotomy site |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | |

The above donor is FIT / UNFIT to donate blood.

Blood Bag : SINGLE / DOUBLE / TRIPLE

Volume : 350 ml /450 ml

Signature of the MEDICAL OFFICER.

# INTRODUCTION

Blood transfusion is an important part of modern medicine and often a life – saving procedure. But it carries the major risk of transmission of infections because of large volume of human source material is infused directly into the body and blood collected from a single infectious donor may be transfused to a large number of recipients.[1]

Transfusion transmissible agents have certain characteristics. They are present in an infectious or potentially infectious form in the blood, and are stable during storage and transmitted via the parenteral route. Most importantly, they can cause asymptomatic infections in the donor and an apparently healthy prospective donor could potentially be harboring infections.

To minimize transmission of the diseases, three levels of safety strategies are instituted.[2]

Level-I: Predonation screening to defer unsuitable donors with risk behaviors.

Level-II: Screening of donated unit for the presence of infectious disease markers .

Level-III: Minimizing blood transfusion to the extent possible and using blood only when truly needed.

In India, as per the drug control mandate, every unit of blood collected should be screened for HBsAg, antibodies to Syphilis, HIV1& 2 and Hepatitis C virus, and malarial parasite. Only units found non-reactive to all mandated tests should be released for issue. All reactive units are to be disposed off as bio-hazardous waste.[3]

Leptospirosis has been recognized as an emerging global public heath problem because of its increasing incidence in both developing and developed countries.[4]

In India leptospirosis has been documented from all over southern India, Maharashtra, Gujarat, Bengal and the Andamans.[5]

The worldwide prevalence of leptospirosis among voluntary blood donors is 1.01%.[5] In India, the prevalence 2.3% In Chennai 2.7% prevalence has been reported.[6]

Leptospirosis is of interest in transfusion medicine because it is possible to transmit and acquire leptospirosis through blood transfusion. Though transfusion transmitted leptospirosis is not mentioned in standard medical, infectious disease, and transfusion medicine literatures, there is some evidence that this may indeed occur.[6]

# AIMS & OBJECTIVES

i. To study the prevalence of leptospirosis among voluntary blood donors in Chennai city

ii. To detect leptospira by the following three methods- Dark Field Microscopy (DFM), Microscopic Agglutination Test (MAT), Polymerase Chain Reaction (PCR).

iii. To find out the feasible screening method for routine use in blood banks if needed.

iv. To detect the optimum survival time of leptospira in plasma stored at -30°C.

v. To assess whether leptospiral screening should be included as a screening for voluntary blood donors.

# REVIEW OF LITERATURE

Blood Transfusion is a life saving procedure. The first human transfusion was performed in France & England in 1667. It was James Blundell who clearly demonstrated the use of human blood for human transfusion. The first well documented transfusion with human blood took place on Sep.26, 1818.[1]

In 1900, Karl Landsteiner observed blood groups A, B, C, (subsequently renamed `O') by Landsteiner and of group AB by Decastello and sturli. It was Reuben Otlenberg (1882 – 1959) who demonstrated the importance of compatibility testing.

Transfusion reactions were well documented and understood by 1950s when blood transfusion practice expanded. Although major and minor reactions occurred, even after the introduction of routine typing and cross-matching. It had been known for some time that blood transfusion, transmitted syphilis, malaria, small pox. In U.S. they suspected syphilis to be transmitted and hence made VDRL as the mandatory test (1942).[1]

It was during 1953, Paul Beeson, described transfusion transmitted hepatitis. Subsequently they found Hepatitis, A, B, C to be transmitted However, it was the outbreak of AIDS that galvanized the public attention to blood transfusion.

Improved donor screening and increased donation testing have greatly decreased the risk of disease transmission and rendered the blood supply safer.

The disease transmission depends on the type of donor, their behavior and risk status. The following are the types of donors.

**VOLUNTARY DONORS**

A person who gives blood, plasma or other blood components of his / her own free will and receives no payment for it, either in the form of cash or in kind which could be considered a substitute for money, this includes time of work other than reasonably needed for the donation and travel. Small tokens, refreshments and reimbursement of the direct travel costs are compatible with voluntary, non – remunerated blood donation. He is an altruistic donor , who gives blood fully and willingly.[2]

**Categories of voluntary blood donor**

1) **New voluntary donor**: A voluntary non – remunerated blood donor who has never donated blood before.

2) **Lapsed Voluntary donor**: A voluntary non – remunerated blood donor who has given blood in the past but does not fulfill the criteria for a regular donor.

3) **Regular voluntary donor**: A voluntary non-remunerated blood donor who has donated at least three times, the last donation being within the previous year and continues to donate regularly atleast once per year.

**Family / Replacement blood donor.[2]**

1) A donor who gives blood when it is required by a member of the patients' family or community. This may involve a hidden paid donation system in which the donor is paid by the patients' family.

2) A family / replacement donor is one who gives blood when it is required by a member of his / her family or community. This often involves concern and / or payment which compromise the safety of the blood.

3) A member of the family or a friend of the patient who donates blood in replacement of blood needed for the particular patient without involvement of any monetary or other benefits from any source.

**Paid / professional donor**

A donor who donates blood in exchange of money or other form of payment is called a professional donor. It is banned in India w.e.f. 1st January 1998.[2]

Voluntary Blood Donors are the cornerstone of a safe and adequate supply of blood and blood products. The safest blood donors are voluntary, non-remunerated. Despite this notion, family / replacement donors still provide more than 45% of blood collected in India.[2]

Many reports confirm that paid donors and replacement donors have a higher prevalence of transfusion transmitted infections.

In a study conducted by Ekadashi, et al., in New Delhi, found that HIV seropositivity and syphilis seroreactivity were more among replacement donors than voluntary donors.[7]

In a study conducted by Gupta et al. (New Delhi) in 2006 among Armed forces, seropositivity of HIV, anti-HCV, HbsAg showed an increase in reactivity rate in replacement donors compared to voluntary donors.[8]

In a retrospective study conducted by Prahalathan et al. during 1996 to 2007, in Madurai, they found that the prevalence of HIV was 6 times more common among replacement donors compared to voluntary donors.[9]

The goal is to wipe off the scarcity of blood and ensure availability of safe and quality blood and other blood components, round the clock and throughout the

year. This will lead to alleviation of human sufferings, even to the far – flung remote areas in the country.[2]

For a safe blood service in our country, where comprehensive tests are neither possible nor pragmatic, it is best to switch over to 100% voluntary donations, which provides the safest blood.

According to WHO estimate 43% of blood collected in the developing world is not adequately screened. This means that about 80% of world population has access to only 20% of the global supply, of safe and completely screened blood. It is therefore critical for developing countries to design their own blood safety strategies adapted to their epidemiological, social and economic circumstances.[1]

The key to recruiting and retaining safe blood donors is done with;

1. Good epidemiological data on the prevalence and incidence of infectious markers in the general population,

2. To identify low risk donor population coupled with an effective donor education,

3. Motivation and recruitment strategy to recruit new voluntary non-remunerated blood donors from the low risk donor population.[1]

Blood donors must meet certain safety criteria to protect both the blood donor and the recipient. The Food and Drug Administration (FDA), and the American Association of Blood Banks (AABB) has laid certain criteria for blood collection, but each organization determines its own criteria for acceptance for numerous diseases, conditions and behaviors that they encounter.

Likewise infectious disease screening also varies depending on each region according to the prevailing local infections.

The latest classification of transfusion transmitted infection is presented in the following table based on the 'Canadian International Medical Reports (2006).'

**Classification of transfusion transmitted infections[10]**

| | **First Group** | **Second Group** | **Third Group** | **Fourth Group** | **Fifth Group** |
|---|---|---|---|---|---|
| Agents | HBV, HCV, HIV-1/-2, HTLV-I/-II, *Treponema pallidum* | CMV, EBV, *Yersinia enterocolitica, Staphylococcus* and *Streptococcus* species, *Trypanosoma cruzi* | HAV, HPV-B19, HHV-6, *Plasmodium* species, *Babesia microti, Rickettsia rickettsii, Leishmania donovani, Toxoplasma gondii* | HGV/ GBV-C, TTV, SEN-V | HEV, HHV-8, *Borrelia burgdorferi, Ehrlichia phagocytophila,Prion* |
| Transfusion transmissibility | Established | Established | Established | Established | Not established |
| Pathogenicity | Cause diseases | Cause diseases largely in high risk individuals | Cause diseases largely in high risk individuals | Not established | Cause diseases |
| Donor serologic test | Available | Not available | Not available | Not available | Not available |

Emerging infectious disease are clinically distinct conditions whose incidence in humans has increased, often within the past 2 decades.

Emergence can be due to the introduction or appearance of a new agent, the recognition of on existing but previously undetected agent or through environmental changes that provide suitable niches for agents to invade.

The following are the factors influencing the emergence of infectious agents.[11]

| Factor | Examples |
|---|---|
| Human demographics + behaviors | Population growth, immunosuppression, sexual activity. |
| Technology + industry | Modern medicine, food processing and delivery, water treatment |
| Economic development and land use | Dambuilding, representation of global warming. |
| International travel and commerce | Aircraft, business travel, relocation |
| Microbial adaptation and change | Natural variation / change, selective pressure, development of resistance |
| Breakdown of public health measures | Inadequate sanitation, complacency, war |

The following are the emerging infections potentially or actually transmissible by blood transfusion – *Prions* (vCJD), Viruses, *Chikunguya, Dengue,* HBV variants, HHV, HIV variants, *Influenza,* SARS, SFV, WNV. Bacteria – *Anaplasma phagocytophilium, Borrelia burgdorferi, Parasites – Babesia spp., Leishmania spp., Plasmodium spp., Trypanasoma cruzi.* [12]

Leptospirosis has been recognized as an important emerging global public health problem because of its epidemic properties and increasing incidence in both

developing and developed countries. It is an active bacterial infection caused by spirochetes, with different pathogenic species of the genus Leptospira. [4]

Leptospirosis has wide geographic distribution and occurs in tropical, subtropical and temperate zones. In the developed world, the incidence of the disease has come down substantially and most cases that occur now are associated with recreational exposure to the contaminating water. Contrastingly, the incidence appears to be increasing in developing countries .[5]

In a study conducted by Shivakumar et al in Chennai, the prevalence of leptospirosis was twice common among people residing in northern part of Chennai. (23%).[5]

Several outbreaks of leptospirosis is reported in various places and in India, some of these outbreaks were associated with natural calamities such as cyclone and flood.[5]

Leptospirosis is a direct zoonosis. They are maintained in nature by a large variety of wild and domestic animals. Leptospires shed in the urine of these carrier animals can survive in the environment, for prolonged periods. The source of human leptospiral infection is infected animal's urine. Hence, the commonly considered risk factors and behaviors are those that expose people to animal reservoirs or contaminated environment. Contact with various species of animals, animal tissue, animal urine and wet environment and occupational and recreational exposure to contaminated water bodies have been implicated as risk factors.[5]

## HISTORICAL ASPECTS

Diseases clinically similar to leptospirosis were recognized as occupational hazards of the rice farmers in ancient China.[13]

Adolf Weil reported his description of a clinical syndrome characterized by splenomegaly, jaundice and nephritis, commonly referred to as Weil's disease which became synonymous with leptospirosis.[14]

## ORGANISM

Leptospira is a spiral shaped bacterium.

They are long (6-20 µm), thin (approximately 0.1µm), tightly coiled spirochetes that are actively motile. The motility is characterized by flexion, extension and rotation about its longitudinal axis. The rotatory movement occurs in both directions alternatively. Usually one or both ends are bent or hooked **(Fig.A.1).** They are too thin to be seen under light microscope and are best visualized under dark field microscope.[20] **(Fig.A.2).**

Straight forms also occur that rotate and travel more slowly than hooked forms. These organisms are not readily stained with aniline dyes and Giemsa stain, but seen well with silver impregnation technique.[20]

It exhibits a surface architecture that resembles gram negative and gram positive bacteria. Double membrane constitution supports gram negative bacteria whereas attachment of peptidoglycan to the inner membrane resembles gram positive nature.

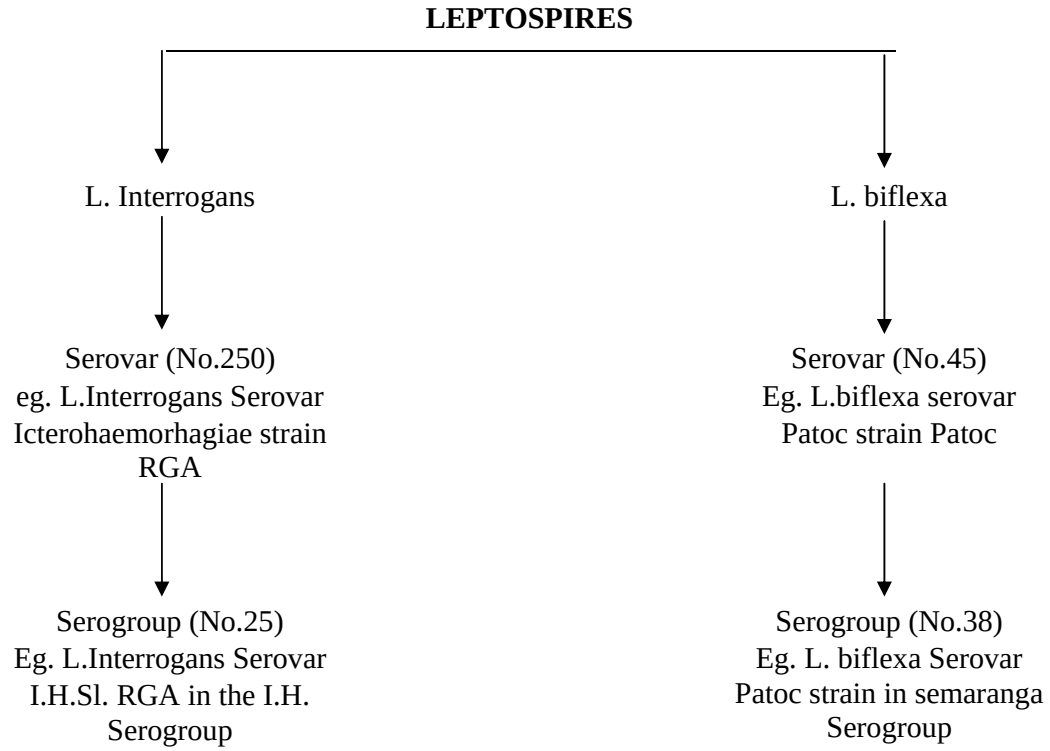
**BASIC TAXON OF LEPTOSPIRES, INTRA SUBSPECIFIC, RANKS-SEROVARS & SEROGROUPS**

Leptospires belongs to Division-Gracillicutes; Class – Scoto bacteria; Order – Spinochaetales and Family – Leptospiraceae which has 3 genera viz., Leptospira, Leptonema and Turnaria.

The classification and nomenclature of Leptospira is complex.

There are two different classification systems - one based on phenotypic characters and other on genetic homology. There are two species namely, L. interrogans (Pathogenic) and L. biflexa (non-pathogenic). [15]

Both the species have several serovars and serovar is the basic taxon, which is defined on the basis of surface antigenic make up .Closely related serovars are arranged into serogroups. Serogroups designation is intended for laboratory use.



268 pathogenic serovars have been published and each one has reference strain.

**PATHOGENIC AND SAPROPHYTIC ORGANISMS**

It can be pathogenic or saprophytic, free – living and generally considered not to cause disease.

Pathogenic leptospires are maintained in nature in the renal tubules of certain animals.

Saprophytic leptospires are found in many types of wet or humid environments ranging from surface waters and moist soil to tap water. Saprophytes are supposed not to cause disease. They are found in cultures when sterility was not maintained during preparation of culture media, when non-sterile ingredients were used for the preparation of culture media or when clinical samples were not collected aseptically. [16]

**ANIMAL RESERVOIRS OR VECTORS**

Leptospires dwell in the renal tubules of their animal host. The transmission cycle of leptospirosis involves the maintenance hosts, the carrier hosts, the environment and human beings. Almost every known species of rodent, marsupial and mammal can be a carrier and excretor of leptospires.[15]

Rats and bandicoots have shown evidence of anti-leptospiral antibodies following isolation of leptospires from the dwelling area of suspected human patient in the suburbs of Chennai (Saravanan et al., 2000). [16]

In Tamil Nadu, anti-leptospiral antibodies (52.1%) were evident from the field rodent (Natarajaseenivasan et al, 2002). Ratnam et al., (1983) screened 40 cows in a

village near Chennai following an outbreak of leptospirosis in cattle. Antibodies against leptospires were found in 68% of cows. [16]

Venkatraman and Nedunchelliyan (1992) reported an outbreak of leptospirosis in humans and dogs in Chennai. Seroprevalence was 50.5% (human) and 21.3% (dogs).[17]

**TRANSMISSION DYNAMICS**

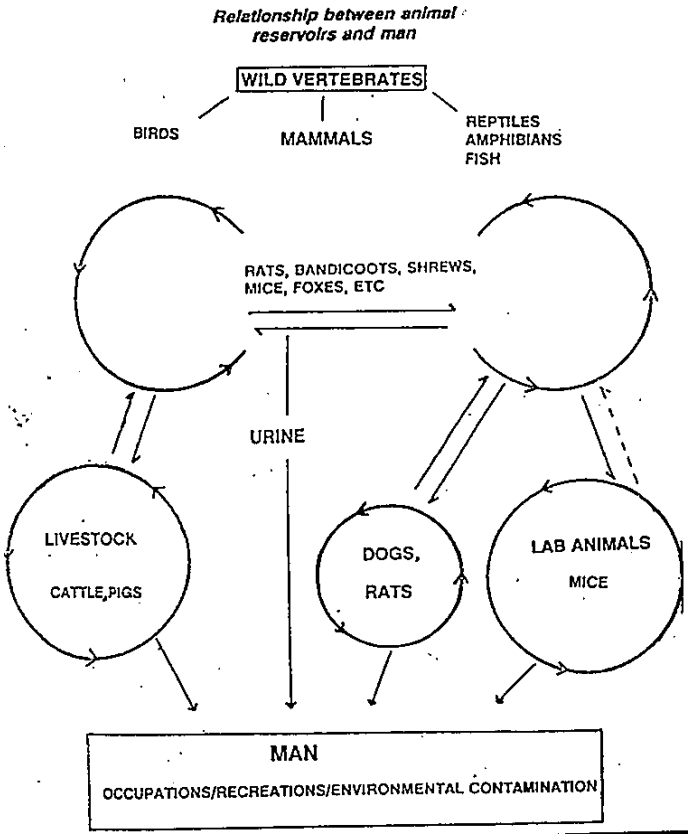
Transmission can be direct or indirect. Direct transmission occurs when leptospires from tissues, body fluids or urine, of acutely infected or asymptomatic carrier animals enter the body of the new host. Diseases are seasonal with peak incidence occurring in summer, where temperature is the limiting factor in the survival of leptospires and during rainy seasons in warm-climate regions, where rapid desiccation would otherwise prevent survival. [16] **(Fig.A.3).**

Direct transmission from animals to humans is common amongst the occupational groups, who handle animals and animal tissue such as butchers, veterinarians, cattle, pig farmers, rodent control workers.[16]

Certain occupational groups are at high risk – veterinarians, agricultural workers, sewage workers,slum people, slaughter house employees and workers in fishing industry.[18] **(Fig.A.4).**

There was a report of seroprevalence rate of leptospirosis – 68.3% among rice mill workers of Salem.[17]

In a study by Bolin and Koellner, Human to human transmission of leptospirosis through breast feeding has been recorded.[19]




Indirect transmission occurs when an animal or human being acquires leptospirosis from environmental leptospires, originating in the urine of excretor animals.[18] They can survive for long periods of time in the environment and probably multiply when conditions are favorable. Most common portal of entry of leptospires into the host body is through the intact skin.[13]

Mishra et al., (2003) reported prevalence is higher in rural, who are handling live stock farm than urban.[4] There is evidence that transmission of L.interrogans by blood transfusion is possible, but to date, there has been no documentation of transfusion transmitted leptospirosis.[6]

## BIOLOGIC SPECTRUM OF DISEASE

### Antigens and Immune Response

Leptospirosis have a complex antigenic structure. The somatic antigen is genus specific. The surface antigen is a polysaccharide and is serovar specific. The outer membrane is a potent immunogen - lipopolysaccharide in nature. It is the major antigen and target of antibody and complement mediated bactericidal activity. Antibodies directed against it are protective in nature. Flagellar antigen is composed of genus and serotype specific antigens. Some serovars, eg L.icterohaemorhagiea, have an additional Vi antigen, associated with virulence.[19]

## ANTIBODIES

Antibodies are directed against

- Common antigens (genus specific antigens) that are shared by pathogenic and saprophytic.
- Serovar specific and serogroup specific antigens.

Patients with leptospirosis may produce antibodies that react with several serovars (phenomenon called "Cross reaction" seen in the initial phase of the disease).[19]

Cross reactive antibodies gradually disappear as the immune response matures in the course of weeks or months, while serogroup and serovar specific antibodies often persist for years. Thus, genus specific antibodies remain detectable for months, serovar- specific antibodies for years. Serovar specific antibodies are protective and the patients are immune to re-infection with the same serovars, as long as the concentration of specific antibodies is high enough. Antibodies provoked by an infection with a particular serovar, do not necessarily protect against infection with other serovars.[15]

The immunologic response to leptospires is both humoral and cell mediated after entry of organism into the host, both 'T' and 'B' cell dependent areas are stimulated .The initial elimination is done by phagocytosis. The antibody response in classical with peak IgM levels appearing first, quickly followed by IgG antibodies, which persist longer than IgM . High IgM levels can be observed during the first 2 months of disease. Heterologous, i.e. genus specific, antibodies appears first. But decline faster. Homologous i.e. serovar specific, antibodies appear later and persist longer.[16]

Recovery from infection in possible after the appearance of lytic and opsonic antibodies and phagocytic clearance of leptospires from blood and tissues.[20]

**PATHOGENESIS**

The most frequent sources of infections are urine, kidneys, surface water, mud and soil. Leptospires are presumed to enter via small abrasions or other breaches of the surface integument. They may also enter directly into the blood stream or lymphatic system via the conjunctiva, the genital tract in some animals, the nasopharyngeal mucosa, lungs and invasion of placenta (in mammals). [13, 19]

Pathogenic leptospira rapidly invade the blood stream after penetrating skin or mucous membrane. The primary lesion in leptospira is disruption of the integrity of cell membrane of endothelial cells lining small blood vessels in all parts of body resulting in capillary leakage and hemorrhages. [19]

Infectious vasculitis is responsible for the major clinical manifestations of the disease, including renal tubule and hepatic dysfunction, myocarditis and pulmonary hemorrhage. Intra to extra vascular fluid shifts secondary to endothelial damage lead to hypovolemia, which complicates renal dysfunction and lead to shock.

Leptospires are able to persist in some anatomically localized and immunologically privileged sites, after antibodies and phagocytes have cleared leptospires from all other sites. The most significant site of persistence is the renal tubule. Leptospires appear in the kidney 2 – 4 weeks after on acute infection.[13, 19]

## CLINICAL FEATURES[15]

Incubation period: 2 – 20 days.

Two clinically recognizable syndromes are

1. Anicteric leptospirosis
2. Icteric leptospirosis

### 1. Anicteric Leptospirosis

It's a self limiting illness occurring in 85 – 95% of cases.

The onset is abrupt and in characterized by fever, headache, myalgia, chills, rigors, prostration.

The first (septicaemic) phase, manifests with conjunctival suffusion, skin rash, hepatomegaly, splenomegaly & muscle tenderness.

The second stage (immune) coincides with appearance of Ig M antibodies. Duration of the immune stage ranges from 4 – 30 days and leptospires are cleared from the blood. Leptospiruria develops and persists for 1 – 3 weeks. Aseptic meningitis, uvetis, iritis, irido cyclitis and chorioretinitis may appear.

### 2. Icteric Leptospirosis (Weils Syndrome):

It is caused by any serotype in its severe form, clinically manifested by fever, jaundice and azotemia.

Renal involvement is common in both anicteric and icteric types. Azotemia, oliguria and anuria commonly occur during the 2nd week of illness. Hypotension, hemorrhage, congestive heart failure, changes in sensorium may occur. All form of leptospirosis begins in the same way and at the start of infection it is not possible to predict the outcome.

**Clinical Features of Leptospirosis[14]**

| **Incubation period** | **Septicaemic phase** | **Interphase** | **Immune phase** |
|---|---|---|---|
| 2-10 days | 4-7 days | 1-3 days | 10-30 days |
| • Bacteria enter the body through cuts or mucosol surfaces. Bacterial flagellae aid tissue penetration | • Abrupt onset of fever, headache, muscle, pain, nausea;<br>• Leprospires isolated from blood, C.S.F. and most tissue<br>• Mostly anicteric 5-10% have jaundice | • Fever and other symptoms resolve temporarily prior to oneset of immune phase. | • Recurring fever and CNS involvement (meningitis)<br>• Primarily humoral response<br>• Antileptospiral antibodies lead to clearance of the organism from most of the tissues except kidney tubules |

**Important Leptospiral Infections**[15]

| Serotype | Disease | Clinical Picture | Animal Reservoir | Distribution |
|---|---|---|---|---|
| Icterohemorrhagiae | Weils Disease | Fever, jaundice, hemorrhages | Rat | Worldwide |
| Canicola | Canicola fever | Influenza like, aseptic meningitis | Dog | Worldwide |
| Grippotyphosa | Swamp or marsh fever | Fever, prostration, aseptic meningitis | Field mice | Europe, Africa, SE Asia, USA |
| Pomona | Swineherd's disease | Fever | Pig | America, Europe, Middle East, Indonesia, Australia |
| Hebdomadis | Seven day fever | Fever, Lymphadenopathy | Field mice | Japan, Europe, USA |
| Fortbragg | Pretibial fever Fortbragg fever | Fever, rash over tibia | Not known | Japan, SE Asia, USA |
| Pyrogenes | Febrile Spirochetosis | Fever | Pig | SE Asia, Europe, USA |
| Bataviae | Indonesian Weil's disease | Fever | Rat | SE Asia, Africa, Europe |
| Hardjo | Dairy farm fever | Fever | Cattle | UK, USA, New Zealand |

Shivakumar et al. (2008) studied the prevalence of asymptomatic leptospiral infection among family members and close contacts of leptospirosis patients who are living in a same environment. They showed 31 families (56.4%) out of 55 showing positivity indicating that 56.4% of families having leptospires in their environment. Their study also screened the high-risk group, the family members who are living with an index case of leptospirosis showing a seroprevalence of 36.5%.[49]

Shivakumar et al. also enumerated the probable causes for the asymptomatic infection.

i) Circulation of pathogenic serovars for limited period in specific areas.

ii) Exposure to infection during childhood and development of protective antibodies.

iii) Host susceptibility varies among individual HLA gene polymorphism have been reported to be associated with the risk of acquiring leptospirosis during an epidemic.

In a study conducted by David Ashford in Nicaragua, they reported that fever may be more frequently absent or mild in leptospirosis infection. In Seychelles, 9% of adult males had laboratory results consistent with recent leptospiral infection with no one reporting current symptoms.In Brazil, 22% of study subjects were positive for IgM antibodies but were asymptomatic.[50]

Hence according to the previous studies leptospirosis could be present in an asymptomatic form in many donors who look apparently healthy and get fit into the medical examination for blood donation. Provision of safe blood to the recipients is our goal and strategy.

Leptospirosis may be transfusion transmitted because of the following 3 factors:

1) Presence of leptospiraemia during the incubation period.
2) Asymptomatic
3) Occurrence of leptospiraemia during convalescence

In the available literature, these 3 factors though described are said to be the exception rather than the rule.[5]

## LABORATORY DIAGNOSIS

Broadly classified into

i) Direct evidence

ii) Indirect evidence

Different methods used in the laboratory are categorized into bacteriological, microscopic, serological and molecular.[20]

## DIRECT EVIDENCE

Demonstration of leptospires or its products by the following methods:

**a) Microscopy**

- Dark field microscopy.
- Phase contrast microscopy.

**b) Staining**

- Silver staining.
- Immune fluorescence.
- Immuno peroxidase.

**c) DNA hybridization.**

**d) Polymerase chain Reaction (PCR).**

## INDIRECT EVIDENCE

Detection of antibodies to leptospirosis

b) Genus specific tests

c) Serogroup or Serovar Specific Test

**a) Genus Specific Tests**

- Macroscopic agglutination test (MaAT).
- Indirect fluorescent antibody test (IFAT).
- Indirect hemagglutination test (IHA).
- Counter Immuno Electrophoresis (CIEP).
- Complement fixation test (CPT).

**Newer Techniques**

- ELISA.
- Microcapsule agglutination Test (MCAT).
- Lepto Dipstick.
- Lepto lateral flow.
- Lepto Dri Dot.

**b) Serogroup / Serovar specific Test**

- Microscopic agglutination Test (MAT)

- **Isolation of leptospires from samples like**
  - Blood
  - Urine
  - CSF.
  - Other body fluids and tissues.

- **Animal inoculation**

**Demonstration of Leptospires**

Leptospires are generally found in the blood during the first 10 days of the disease. They are excreted in the urine after 1 week of onset of the disease. Leptospires can also be found in other body fluids such as CSF, aqueous humour amniotic fluid.[22]

**DARK FIELD MICROSCOPY (DFM)**

**Principle**

The specimen is illuminated only by light that are scattered by objects, eg. Bacteria of different refractive index from the suspending medium enter the objective and reach the eye, so that these objects appear brightly against a dark background.

Approximately $10^4$ leptospires per ml are necessary for one cell per field to be visible by DFM.[13,21]

Wolff noted that DFM after differential centrifugation of Ruys, may enhance the chances of seeing Leptospira and thereby make an early diagnosis possible.[22]

But microscopic examination of tissues or body fluids is not recommended as a single diagnostic procedure ,since the concentration of leptospira in the blood may be too low and artifacts such as fibrils and extrusions from cellular elements can be easily mistaken for leptospira by the inexperienced.[13]

Chandrasekhar an et al used 1% solution of liquoid in sterile saline and phosphate buffered sodium oxalate solution at pH 8 to demonstrate leptospira in varying concentration in the blood of patients and police dogs.[5,20,22]

A quantitative buffy coat method has been shown to have a sensitivity of approximately $10^3$ leptospires per ml.[22]

Microscopy of blood is of value only during the first few days of acute illness, when leptospiraemia occurs.

The following are the advantages of DFM. It is the most rapid method of clinching a diagnosis, extremely sensitive when used properly i.e. with a combination of the ideal specimen together with accurate timing of specimen collection, good

microscope and the trained observer. It is relatively inexpensive. It is useful to observe leptospires in cultures and to observe agglutination in the MAT.[22]

The following are the disadvantages of DFM. It has low sensitivity and specificity. Serum proteins and fibrin strands in blood resembles leptospires. It needs technical expertise.

## STAINING METHODS

### Silver Deposition Techniques

Leptospires in smears of tissue or fluids on slides can be stained using silver deposition methods. Well - stained preparation show black spirochetes in pale yellow or brown tissue elements.[19]

### Immunofluorescence

It is preferable to do silver staining, because it is easier to see leptospires in small numbers and the serovars or serogroups can be determined presumptively.[19]

### Serological Tests

Detection of antibodies is by itself no proof of a current infection as some antibodies may persist for long periods after an infection.

Generally, seroconversion (first sample, no detectable titre, second sample – positive i.e. above the cut off point) or a four fold or higher rise in titre (first sample, low titre, second sample much higher titre) in consecutive serum samples is considered to be diagnostic proof of recent or current infection.[15]

**MICROSCOPIC AGGLUTIONATION TEST (MAT)**

The reference method for serological diagnosis of leptospirosis is MAT, in which patient sera are reacted with live antigen suspensions of leptospiral serovars. After incubation, the serum – antigen mixtures are examined microscopically for agglutination and the titers are determined.[21]

The MAT is read by DFM. The end point is the higher dilution of serum at which 50% agglutination occurs. Because of the difficulty in detecting when 50% of leptospires are agglutinated, the end point is determined by the presence of approximately 50% free, unagglutinated leptospires compared to the control suspension. Different laboratories use different cut off titers ranging from 1 in 100 to 1 in 800 for diagnosis and may result in overdiagnosis and overestimation of the disease burden.[21]

Cross–reactions may occur between different serogroups, especially in acute phase samples. Paradoxical reactions, in which highest titers are detected to a serogroup unrelated to the infecting one, are also common.

The MAT is a complex test to control, perform and interpret. Live cultures of all serovars required for use as antigens need to be maintained.

Other drawbacks of MAT include the continuous risk of cross-contamination of the antigen cultures, necessitating periodic verification of each serovar. MAT titers are affected by the culture medium in which the subcultures of large number of strains present hazards for laboratory workers.[21]

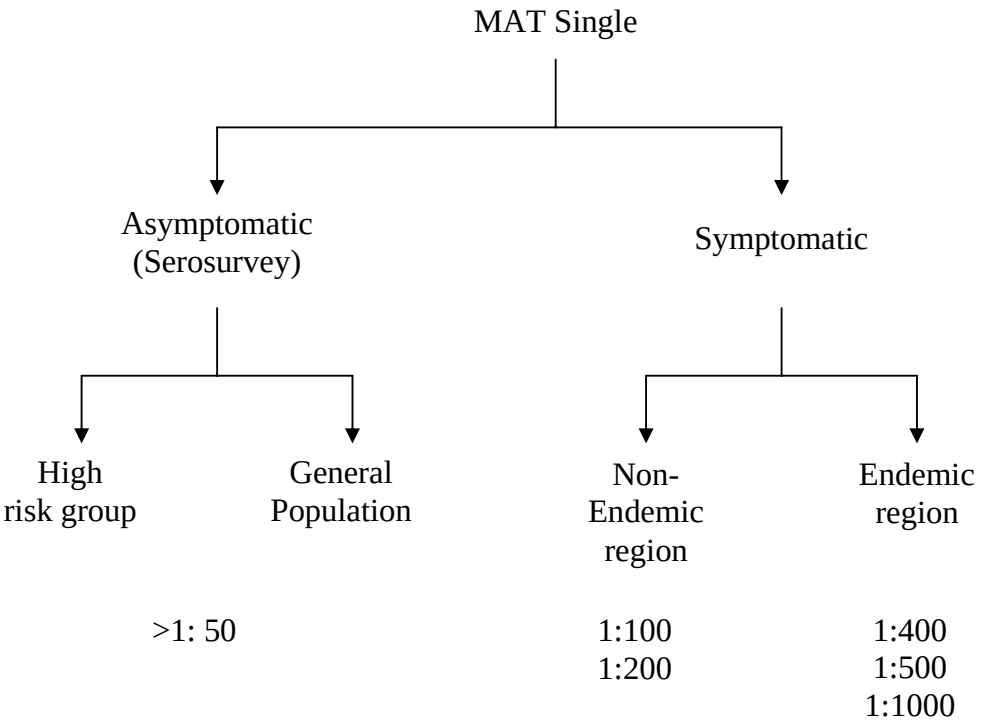
The MAT my reveal the presumptive serogroup to which the causative serovar may belong based on agglutination with different serovars in a panel representative of the locally circulating serovars. Thus, seroepidemiological investigations of serum

samples from the general population may indicate circulating serogroups since residual antibodies from past infections tend to react with serogroup specified antigens.[15]

In 1997, 100 % correlation of a macroscopic slide agglutination test (MSAT), using 3 serogroups with MAT was reported.[19]

A four – fold rise in titer or seroconversion is the most definitive criteria for the diagnosis of leptospirosis Therefore, a second sample is mandatory which is difficult to obtain. Hence, a single high titer in MAT can be taken as diagnostic criteria.[23]

According to Shivakumar et al. a titer of 1: 100 is taken as significant criteria. In endemic areas, a titer of 1/100 or 1/200 is considered low; while a titer of > 1:400 is considered high titer.[10] Some consider a titer of 1:800 or 1:1600 as diagnostic criteria.[23]



**INTERPRETATION OF TESTS**[23]

| ELISA/SAT | MAT | Interpretation |
|---|---|---|
| +ve | Single High Titer | Current Infection |
| +ve | -ve | Current Infection |
| -ve | Single High Titer | Past Infection |
| ± | Seroconversion / 4 fold rise in titer | Current Infection |

Serosurvey on the asymptomatic high risk group, should be done with MAT only and a titer of > 1/50 can be taken as cut off titer.[23]

For studying the prevalence and incidence of leptospirosis, the following can be done ,

1. Serosurvey of asymptomatic high risk groups utilizing MAT.
2. Evaluating the cut off titers of single high titer and determine the serogroups utilizing MAT in samples with positive ELISA/SAT.

**III. POLYMERASE CHAIN REACTION (PCR)**

PCR is used to detect leptospiral DNA in clinical samples. Primers (short DNA sequences that are specific for leptospires) in combination with heat – stable DNA polymerase, in the presence of nucleotides when subjected to temperature cycles, amplifies the target leptospiral DNA. **(Fig.A.5).** PCR can be applied to blood, urine, CSF and tissue samples (ante or postmortem).

PCR has also been used to distinguish pathogenic from non-pathogenic serovars. A fluorescent probe 5' exonuclease PCR assay has also been described for the rapid detection of pathogenic leptospires.[21, 24]

PCR has the advantage that it does not require the isolation of the organism and detects DNA from both viable and non-viable organism.[24]

PCR is simple, specific and rapid method for detection as well as differentiation of leptospires when compared to conventional methods.[4]

PCR could detect leptospiral DNA about 1 or 2 days post – infection, while antibody could be detected from blood after 7 days post infection and culture can detect 7 to 30 days post infection.[15]

Van Eys et al (1989) developed PCR for detection of leptospires in urine samples of infected cattle. Urine samples containing as few as 10 leptospires per ml which gave positive results in PCR assay. Grave Kamp et al (1993) reported that two sets of primers (G1 & G2 and B641 & B651) derived from genomic DNA libraries of leptospiral serovar icterohaemorrhagiae and amplified target DNA of leptospiral species by PCR. They yielded a PCR product of 285bp length and could detect even 1 – 10 leptospires per ml of urine.[5]

Merien et al. (1993) detected leptrospiral DNA by PCR from aqueous humor of a patient with unilateral uveitis. They purified the DNA from 50 micro litre of aqueous humour using silica particles and guanidine thiocyanate method. They amplified a 331 bp fragment by PCR and confirmed by dot blot hybridization with a 289 bp digoxigenin labelled probe internal to the amplified product.[5]

Savio et al (1994) developed an assay for identification of L.interrogans by PCR coupled with REA of amplified DNA fragment.[5]

Brown et al (1995) examined 71 blood and urine samples from acute leptospiral infection by PCR, culture and serology. PCR detected 44 cases (62%) and 34 (48%) were detected by culturing. They reported that the PCR detected leptrospires from 13 patients before development of antibodies. They concluded that PCR was a rapid, sensitive, specific means of diagnosing leptospiral infection, especially during the first few days of the disease.[5]

In another study of 200 patients, suffering clinically with leptospirosis, bacterial culture and MAT showing positivity (Merien et al, 1995), they concluded that the PCR was an efficient tool for diagnosis of leptospirosis during the first 10 days of disease.

Ramadass et al (1997), compared DFM and PCR, for detection of leptospires from clinical samples and found that DFM detected 32% positivity, while PCR detected 73% from 170 samples analyzed by both methods.[5]

Though PCR technique is useful for sensitive and specific detection of pathogenic leptospires form clinical samples, it is of no use in serovar identification.[21]

Leptospiral genomospecies can be differentiated following PCR by electrophoresis in non-denaturing polyacrylamide gels, followed by silver – staining without the additional step of purification and denaturing.[21]

Among 60 negative MAT samples, 8 (13%) was found to be positive by PCR. PCR was positive in 39% patients before the development of antibodies. Use of PCR in adjunction to MAT, the leptospirosis cases increased from 23% to 30% (Kosstanont et al, 2005).[26]

Ramadoss et al (2001) studied the use of arbitrarily primed PCR method (AP-PCR) for typing of leptospiral serovars from direct serum culture and was found to be a good and sensitive method.[27]

Sugathan et al (2005) has shown the use of multiplex PCR using both the primer pairs in diagnosing leptospirosis and additionally in delegating them to the L. Kirchneri or non-L.Kirschneri groups.[28]

# MATERIALS & METHODS

## STUDY PLACE AND DESIGN

A prospective study conducted amongst voluntary healthy blood donors over a period of 2 years from 2008-2009 in the Department of Transfusion Medicine at The Tamilnadu Dr.M.G.R. Medical University, Guindy, Chennai-32.

**Sample Size** : A total of 100 voluntary healthy blood donors Consisting of 10 donors each randomly selected from 10 camps conducted at different regions of Chennai.

**Inclusion Criteria** : All Voluntary healthy blood donors, both Males and females in the age group of 18-65 years.

**Exclusion Criteria** : Blood donors who were not willing to give consent to participate in the above study.

## SAMPLE COLLECTION

5 ml of blood was collected directly from the donor in a sterile plain test tube and allowed to clot; serum was separated and stored at $4^0$ C and $-20^0$ C in two separate aliquots for MAT and PCR studies respectively.[13] Another 5 ml of blood was collected from donor's blood bag immediately after collection and used within 4 hours for DFM study.[15]

## STATISTICAL ANALYSIS

Statistical analysis was done with "Chi-square Test" using statistical software packages (Microsoft Excel, SPSS10). Groups were assumed to differ significantly when the probability (p value) was less than 0.001 (1% Level) or 0.05 (5% level).

## METHODS

### 1. DARK FIELD MICROSCOPY (Fig.A.6).

Plasma samples separated from the 5ml of donor's collection bag blood were examined immediately within 4 hours and the positive samples were kept at $4^0$ C and further examined at 24 hrs, 48 hrs and 96 hrs to assess the survival time of the organism. Segments from the fresh frozen plasma stored at $-18^0$ C of the same samples were also examined at 24 hrs, 48 hrs and 96 hrs.

**Procedure**

- Centrifuged 5 ml of blood at 1000g for 15 mins.
- Added 10 µl of plasma on a thin microscopic slide and applied coverslip
- Examined under dark field microscope with low power and high power (20X and 40X)
- If no leptospires were seen, the plasma sample was centrifuged at 3000-4000g for 20 min., the supernatant was removed carefully and a drop of sediment was re-examined microscopically as above.

**Interpretation**

Motile spirochetes with characteristic motility and rotation pattern of leptospira against dark background were considered positive. **(Fig.A.7).**

### 2. MICROSCOPIC AGGLUTINATION TEST (MAT)

**Material**

- Dark field microscope.
- Battery of young (7-10 days old) live leptospiral antigen grown in liquid media.

- Perspex microtitre plate
- Phosphate buffered saline pH 7.2
- Test sera.
- Test tubes for sera dilution.
- Micropipette 25 µl
- Disposable micro tips
- Glass slide, cover slips
- Sterile gloves

**SEROVAR PANEL**

- Australis
- Autumnalis
- Canicola
- Griptotyphosa
- Hardjo
- Icterohaemorrhagiae
- Louisana
- Pomona
- Pyrogens
- Patoc
- Tarassovi
- Bataviae

**Procedure**

- The sera were diluted to 1:10 in separate test tubes.
- 25 µl of PBS pH 7.2 was added to all the wells in the microtitre plate
- The sera were diluted by doubling as 1:10, 1:20, 1:40 etc. and further diluted upto 1280.
- 25 µl of antigen was added to each well in the row and the dilution of the mixture was doubled as 1:20 to 1:2560 (each row of the microtitre plate was used for each serovar from the panel).
- Control of antigen was put up simultaneously.
- The serum antigen mixture was mixed by gentle agitation and it was kept at room temperature for 2 hours.
- One drop from each well was examined in a slide from lower dilution onwards after examining the antigen control.
- By dark field microscopy, agglutinated masses and free organisms were looked for.

**Interpretation**

The highest dilution of serum antigen mixture, which shows 50% agglutination, is taken as the end titre of the serum for that particular antigen.[20] **(Fig.A.8).**

**POLYMERASE CHAIN REACTION**

**Materials**

- DNA extraction kit (Medox Biotec India)
- PCR master mix (1.5mM MgCl2)
- 1Kb DNA ladder (0.5-10 kb DNA ladder)
- Gel loading dye
- Set of primers (specific for leptspire)

  5' CGC TGG CGG CGC GTC TTA AA 3'

  3' TTC ACC GCT ACA CCT GGA A 5'
- Tris Borate Buffer (0.5%) (TE)
- Double distilled water
- Ethidium bromide (5µg/ml)
- Agarose (2%) (molecular biology grade)

**Equipments**

- PCR thermal cycler (PTC 200, MJ Research) **(Fig.A.9).**
- Microcentrifuge
- Horizontal gel electrophoresis apparatus
- UV Transilluminator
- Gel casting platform with comb.
- Eppendorf microcentrifuge tube
- PCR tube
- Vortex mixture
- Dry heating block

- Micropipettes and sterile microtips
- Sterile gloves
- Tissue paper, permanent marker pen.

**Procedures**

- Serum stored at –20°C is brought to room temperature.
- Kits and reagents were brought to room temperature.
- DNA extraction done by using the extraction kit by Medox Biotech, India.

**Amplification**

- The reaction mixture 50 µL contain

| | | |
|---|---|---|
| 10 µL | - | Master Mix (0.5 mM MgCl2) |
| 1 µL | - | Forward primer |
| 1 µL | - | Reverse Primer |
| 5 µL | - | Template DNA |
| Remaining | - | Distilled water |

Amplification was carried out in a thermal cycler (PTC 200, MJ Research) using 0.5 ml thick microfuge tubes.

PCR Profile was as follows:

| | | | |
|---|---|---|---|
| i) | 94°C | - | 4 Min. |
| ii) | 94°C | - | 1 Min |
| iii) | 55°C | - | 1 Min. |
| iv) | 72°C | - | 1 Min. |
| v) | Go to step (ii) for 34 cycles | | |
| vi) | 72°C | - | 5 Mins |
| vii) | 4°C | - | infinity |

Reaction products (10µL) were subjected to electrophoresis on 2% agarose gel using Tris borate buffer (pH8.3) containing ethidium bromide (0.5µg/ml).

In the agarose gel, the first well was loaded with 1 KB DNA ladder, the second one with a positive control. The amplified DNA samples were subjected to electrophoresis after being loaded in the subsequent wells. **(Fig.A.10).**

**Interpretation**

After electrophoresis, reading was taken in a UV transilluminator. The samples which showed a band at 285 bp corresponding to the positive control were considered as positive. **(Fig.A.11).**

# RESULTS

In the present study, the most common age group of voluntary blood donors was between 21 to 30 yrs (45%), followed by 18% (31-40 yrs), 17% (41-50 yrs), 10% (18-20 yrs) and 10% (51-60 yrs). **(Fig.1.B, Table.1)**

Sex distributions of the donors were 75% males and 25% females. **(Fig.2.B, Table.2)**

In our study, the percentage distribution of voluntary blood donors on the basis of their occupations were 43% professionals, 26% clerical job workers, 16% students and10% Coolies. The remaining 5% were unemployed. **(Fig.3.B, Table.3)**

Based on the residential addresses provided the zonal distributions of the donors participated in this study were, 11% from zone 9, 20% from zone 10 and 26% from outside Chennai. **(Fig.4.B, Table.4)**

Of the 100 plasma samples 31 were positive by DFM study at the time intervals of 4hours after collection at room temperature, and 24 hrs & 48 hrs after storage at $4^0$C. Subsequent examination at 96 hrs the samples were negative by DFM. **(Table 5,6).**

The whole blood collected from the study group was separated into components. The100 fresh frozen plasma samples thus prepared were tested under DFM after 24 hrs of storage at $-18^0$C, none of the samples were found to be positive.

MAT test was performed on all the samples irrespective of the DFM results.

Of the 100 serum samples 40 were positive by MAT. The serovars were Patoc (37%), autumnalis (32%), australis (21%) and Canicola (11%) icterohaemorrhagiae (5%). (**Fig.5.B, Table 7).**

21 % of the donors were positive by both DFM and MAT. The serovars in these samples were autumnalis (38%), patoc (14%), australis (29%), canicola (10%) and Icterohaemorrhagiae (9%) **(Fig.6.B, Table 5,6 ,8 ).**

10% of the donors were positive only by DFM. (**Table 5,6 )**

Of the 40 samples positive for MAT, 19% were negative by DFM. The distribution of serovars were autumnalis (32%), patoc (37%), australis (21%), canicola (10%) **(Fig.7.B, Table.5,6, 9).**

In 50% of donors, both DFM and MAT were negative**. (Table.6)**

The distribution of the samples positive for DFM alone were 29% between 21-50 yrs, 6.5% each in 18-20 and 51-60 yrs. **(Fig.9.B, Table 11).**

The distribution of the samples positive for MAT alone were 32.5% between 21-30 yrs, 30% between 31-50 yrs, 5% between 51-60 yrs and 2.5% among 15-20 yrs**. (Fig.10.B,Table.12).**

Of the 40 donor samples positive for MAT, the zonal distribution in chennai were 20% from zone 10 and 23% from outside Chennai **(Fig.12.B, Table 14)**.

In the study of relating occupation with MAT positivity the following were the distribution – 45% professionals, 33% clericals, 15% coolie, 5% by students, 3% by unemployed. **. (Fig.B.11, Table.13).**

2 donors who showed DFM and MAT positive were also positive for HBsAg. **(Fig.B.8 Table.10).**

All samples which were examined by PCR were found to be negative**..**

# DISCUSSION

In the present study, the most common age group of voluntary blood donors were between 21-30 years (45%) with sex distribution of 75% males and 25% females.

In our study, Maximum number of donors was professionals accounting to 43% of the total donors. This could be explained by the fact that majority of blood donation camps were conducted only in leading professional IT companies, government offices, engineering colleges, and various non-governmental organizations by our blood bank. This could also be explained by rapid urbanization in Chennai, where there is an upsurge of software companies, industries during this decade.

As our blood bank adheres strictly to 100% voluntary blood donation, all the donors included in the study were Voluntary blood donors. Hence, the prevalence of Transfusion Transmitted Infections among our donors was low. Ekadashi, et al., from New Delhi, reported that HIV seropositivity and syphilis seroreactivity were more among replacement donors than voluntary donors.[7]

Gupta et al also reported increased seropositivity of HIV, Anti HCV, and HBsAg in replacement donors when compared to voluntary donors.[8]

In a retrospective study conducted by Prahalathan et al. during 1996 to 2007, in Madurai, found that the prevalence of HIV was 6 times more common among replacement donors compared to voluntary donors.[9]

In our study, by DFM 31% of voluntary blood donors' samples showed organisms with characteristic pattern of motility suggestive of leptospires. Similar

study by Saranya narayan ( 2001) among 14760 units collected over a period of 5 years showed 4.2% positivity.[34] The higher percentage of DFM positivity in our study was not limited by any seasonal preference in sample collection. The study by Pappachan et al showed 282 out of 340 suspected cases positive for leptospirosis. Their data showed that the leptospirosis occurs throughout the year although the number may increase during the monsoon season (June to January). [29]

In our study, DFM showed positivity in the first 24 and 48 hrs of sample collection followed by absence of live leptospira later on. This was consistent with the fact that all spirochaetes lose its viability in blood stored at refrigerated temperatures, their maximum survival is for 96-120 hours.[33]

In our study, by MAT 40% of the samples were positive with dilution titre of 1:160. The study conducted by Ratnam et al on 25 cases of clinically suspected cases showed 4 fold rise in titre on repeat sampling in 9 cases. The commonest serovar found in our study was autumnalis (35%)

In a study conducted by Lettierei et al. in 2004, 1.4% blood donation samples from US army soldiers showed positivity.[40]

M.A. Ribeiro et al. (1999), found a prevalence of 1.01% positivity, using MAT, IgM ELISA among 2368 healthy blood donors.[6]. In 1995, 30.5% of apparently healthy individuals tested positive for anti-leptospira antibodies by MAT in Cordillena province of Bolivia.[32]

In a study by Bovet et al on random sample of 1067 persons in Seychelles showed a sero-prevalence rate of 37% in the year 1999, whereas 54% sero-prevalence rate was observed among healthy population from North Andaman, Andaman and Nicobar archipelago by Murhekar et al.in the year 1998. Consequent to an outbreak of bovine leptospirosis in Chennai, serological evidence of leptospirosis

was more evident among human. [43] .Also there was a high prevalence of leptospiral antibodies in humans was reported from Somalia in 1982.[19] The results of high seroprevalence rate of leptospirosis in these studies among healthy population is similar to our study.

In our study among 40% of the MAT positive donors, predominant population of 18(45%) donors were professionals, followed by 13(32%) were clericals. The reason for these high prevalence rates among professionals and clericals in our study might be due to the maximum proportion (69%) of the donors belonging to these two groups. Our study result of occupational preponderance is contrary to the studies done by S.Ratnam et al, Sumathi Gnanadesikan et al and Nataraja Seenivasan et al. In a study done by S.Ratnam et al on 584 corporation conservancy workers ( 1993) showed 192(32.9%) workers positive for agglutinins to L.interrogans. In a study conducted by Sumathi Gnanadesikan et al., among 650 conservancy workers, 107 (16.4%) were found to show the presence antibodies in their serum samples. In a similar study involving abattoir workers in which 11% had leptospiral antibodies.[39] In a study conducted by Nataraja Seenivasan et al., in 2002, the seroprevalence among urban population in Madurai was 33.9% comprising of 24.8% conservancy workers, 17.6% agricultural workers. In an another study, Seroprevalence among rice mill workers in Salem District was high (68.3%).[17]

In our study all the 100 samples from voluntary blood donors were subjected to PCR, none of the samples showed a positive reaction. In a study by Gravekamp et al and PD Brown et al on clinical samples from proven leptospirosis patients, the detection rate by PCR was only 50% and 60% respectively. The possible reasons given by them for high number of failure rate in PCR detection of leptospirosis patients was due to the fact that most of the sera used in their study were stored for

considerable time at 4°C.Sera on repeated freezing and thawing might have had led to DNA degradation..

Gravekamp et al, (1995) showed PCR's failure to detect leptospiral DNA is leptopirosis patients. PCR may fail when there are inhibitory factors present in the sample that impede the amplification process, as well as when leptospires are present in very low numbers below the detection level of 1-10 leptospires / ml. The low sensitivity of PCR may be attributed to the lower number of leptospires in the blood of patients with less severe infections.[44]

There are many references about inhibition of Taq DNA polymerases by several factors such as chelation of free magnesium ions, hemoglobin, bile salts, acid polysaccharides from glycoproteins and extreme pH variations. Phenol and Chloroform, used for DNA extraction are also considered to be inhibitors (Paulo M.A. Luchesi et al, 2004). He also found the lysis of bacteria during storage and loss of DNA with the supernatant after centrifuging to concentrate.[48]

The other reasons for negative results in PCR in our study, even after proper standardization might be as follows;

1. Prolonged storage of the samples at –20°C which could have denatured the DNA.

2. The primers used might not have matched the leptospiral DNA present in the samples, because of the fact that leptospirosis have diverse serogroups.

Sumathi et al subjected 110 samples from normal healthy individuals for PCR, which gave positive amplification band in 2 samples, which subsequently showed positivity for IgM ELISA, Dipstick Immuno Assay, Latex Agglutination test, MSAT. It is probable that these two persons had asymptomatic leptospirosis

The diagnostic accuracy of DFM in leptospirosis was evaluated by Vijayachari et al showed a sensitivity of 40.2%, specificity of 61.5%, a positive predictive value of 55.2%.[44] In our study, of the 31 samples positive for DFM 21showed MAT positivity.[31]

Chandrasekaran et al. found DFM showing greater sensitivity of 93.3% (56/60) than that of ELISA 13.3%. It was observed that positivity of DFM decreased from 100% (15/15) to 90.9% (10/11) with increase in the duration of infection for more than one week. He also inferred that motile leptospira would help early and rapid diagnosis. There was also persistence of leptospira in blood of 92.9% cases on repeat testing of 42 cases.[22] In another study he found 54.5% in PUO cases by DFM. He also reiterated that even though artifacts occur, these can be differentiated from leptospira by looking for the flexous elongated forms of varying length (5µm-30µm). 50-100 hpf should be examined before reporting as negative for leptospira. False negativity is possible if the concentration of leptospira is very low, but false positivity cannot occur in the hands of experienced persons.[36]

In 10% of the donors, in our study, DFM alone was positive, while MAT was negative. This might be because, of recent acute infection where the antibody level would not have started to rise. DFM negativity in 19 of the MAT positive samples might be attributed either to the absence of disease in the donor, or seroconversion has occurred with the clearance of leptospira from blood and production of antibodies that remain detectable for months or years, at a low titre.[13]

However, DFM is a cost-effective and rapid technique which can help in the early diagnosis and management of patients.

The observation of a significant titre of MAT in a single sample doesnot necessarily indicates current disease as it may be attributed to persisting antibodies of

a past infection. In endemic areas, persisting antibodies may be found in a large proportion of population. In our study 40% of the samples were positive for MAT.

A negative MAT does not exclude current leptospirosis if the considered serogroup is not available.[23]

MAT has some disadvantages such as technical complexity, high cost and the need to maintain live strains of different serovars with an associated risk of infection to the professionals performing the test .[43]

However, MAT remains very useful for epidemiologic studies, identification of strains, assessment of the probable infecting serogroup, and confirmation of illness for public health surveillance.

It was an incidental finding in which two of the samples positive for DFM and MAT, also showed HBsAg positivity. The significance is not known.

From our study, DFM was found to be cheap, inexpensive and rapid for detection of leptospira, compared to the MAT and PCR which needs sophisticated instruments, reagents, and laboratory.

There are no supporting literatures which have done extensive studies on survival and viability of leptospira in various blood components.

Because of cost constraints of PCR reagents and kits, the number of samples was restricted to 100 in our study.

Blood safety is an essential part of blood banking. Provision of safe blood lies not only on the altruistic behavior of donor, but also depends on the careful laboratory screening methods. As new TTIs constantly emerge day by day, it is the duty of the transfusion medicine physicians to screen and provide safe blood to the recipients. As

from our above discussion, leptospirosis might be present in the blood during the asymptomatic phase which either might have got aborted because of the immune system of the donor or it might have clinically manifested after the incubation period.

Also, the survival of leptospirosis in the recipient blood and its disease causing virulence in the recipient remains a question. Hence, the Pandora box of leptospirosis in transfusion medicine can be opened up in future with more extensive studies regarding the survival time of leptospires in whole blood, packed cells, FFP, platelets individually, and also about the effects of storage on the organism's survival.

**Futurology**

- To extend the project with more number of samples
- To use serovar specific primers.
- To follow-up the recipients who have received blood from the positive samples and the asymptomatic positive donors for the disease manifestation.

# SUMMARY & CONCLUSION

- Prevalence of leptospirosis among voluntary blood donors in our study was found to be 21%.
- Of the 100 samples studied, 31 were positive by DFM, 40 by MAT, 21 by both DFM and MAT; however, none of the samples were found positive by PCR.
- Leptospira survived for 48 hrs in stored plasma at 4-6°C and 24 hrs in fresh frozen plasma stored at -18$^{o}$C.
- DFM technique can be used as a simple, rapid and inexpensive means of leptospira detection.
- Leptospiral screening can be made mandatory in endemic regions like Chennai after undertaking a larger study.

**Fig.13 : Zonewise distribution of MAT positive donors (n=40)**

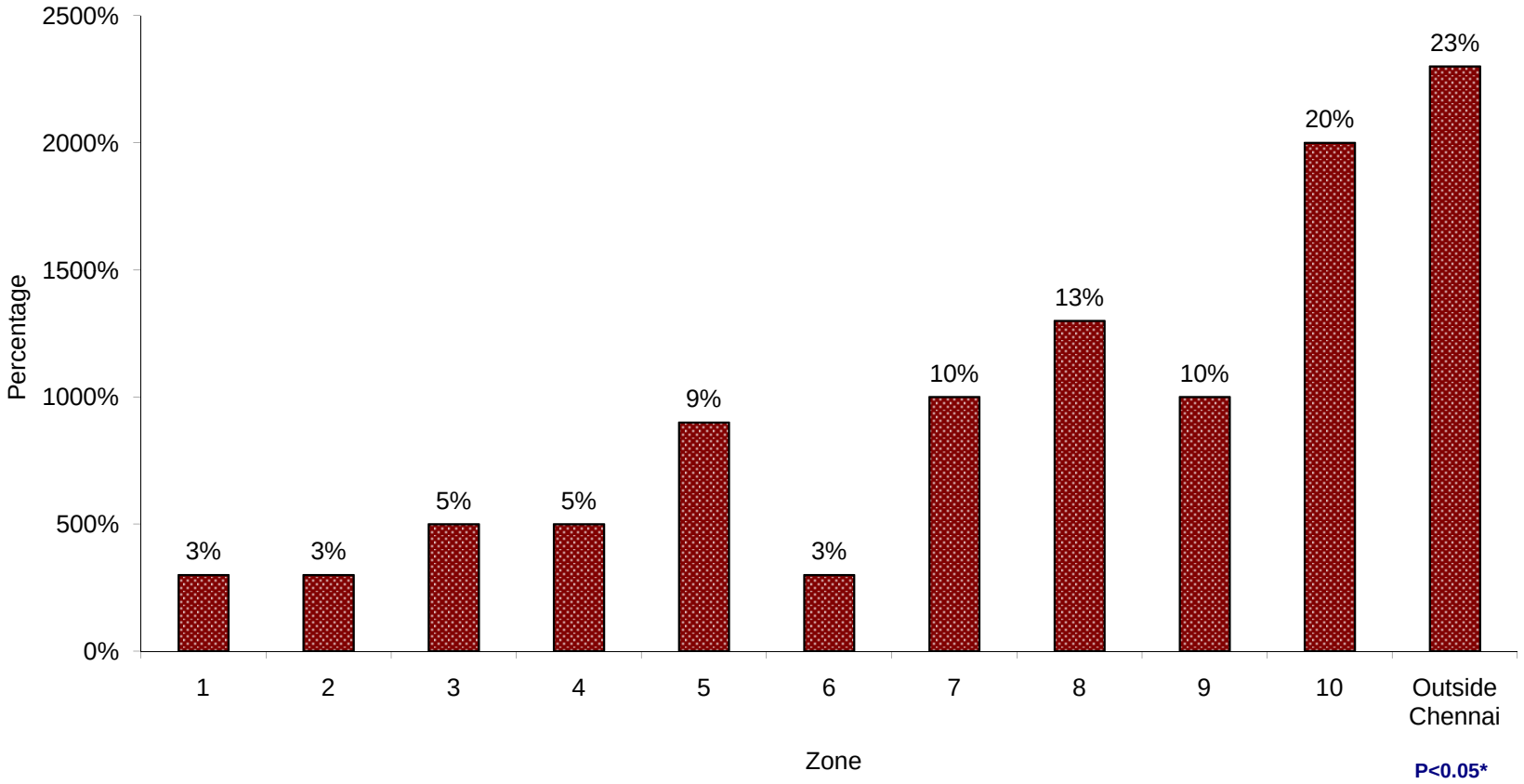
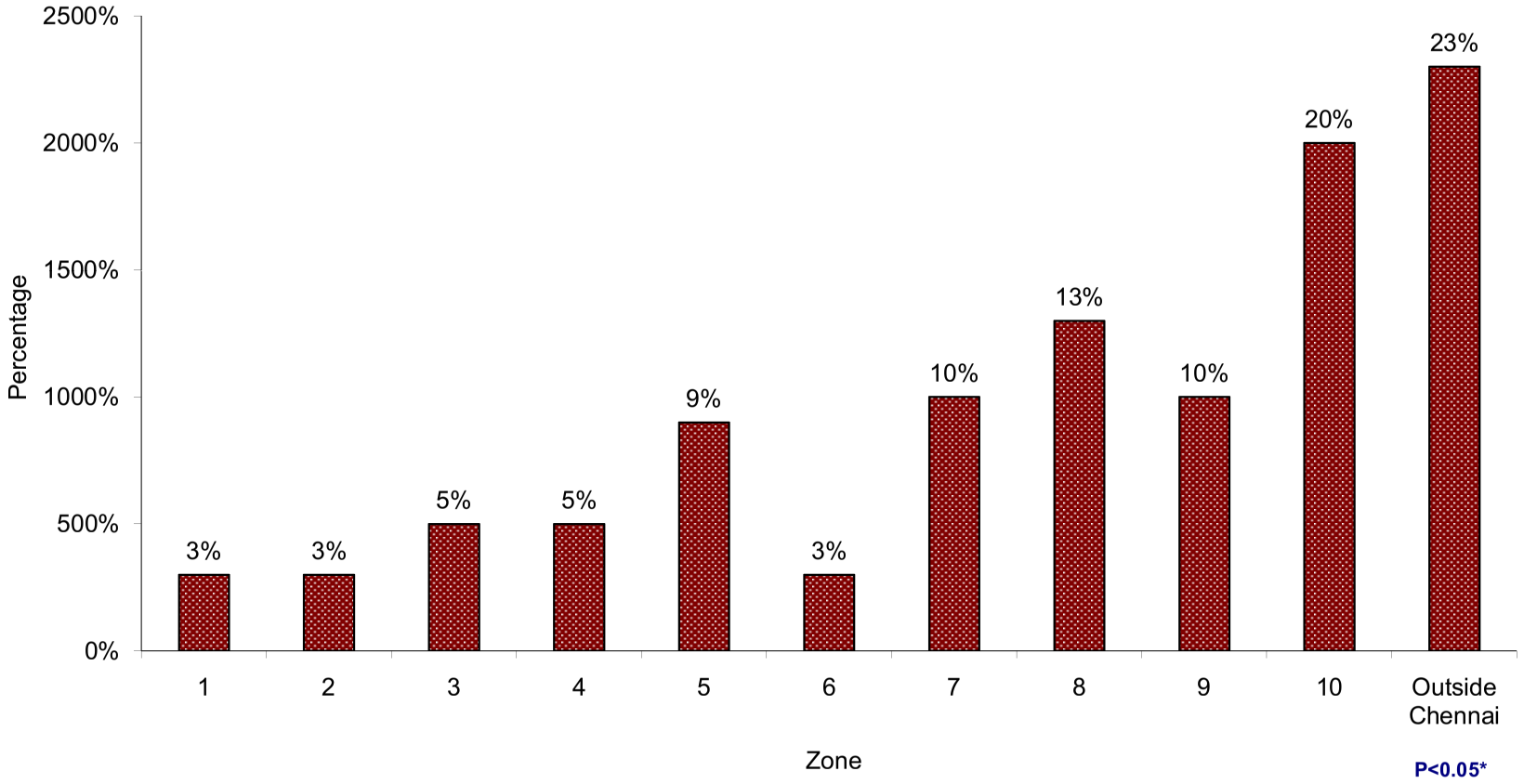


**Fig.13 : Zonewise distribution of MAT positive donors (n=40)**



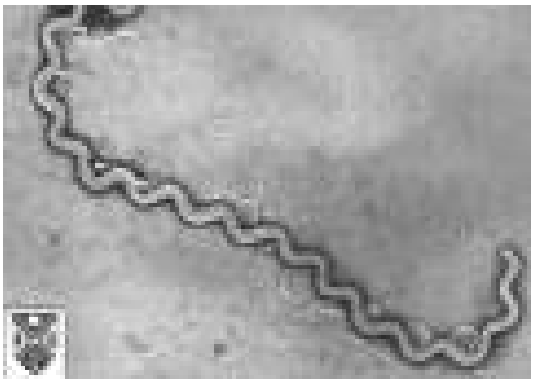


**FIG.A.1:**

**Electron Microscopic picture of a spiral leptospira**

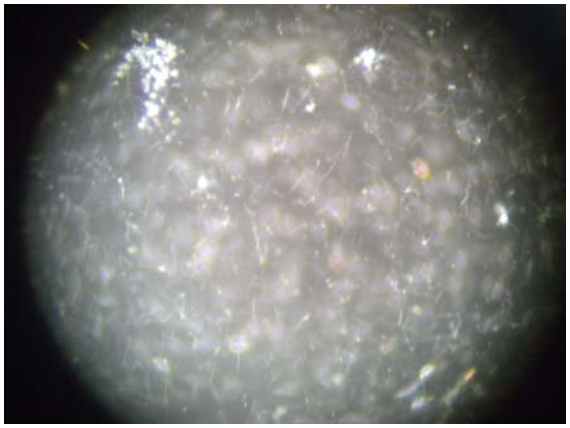


**FIG.A.2:**

**Live leptospires under dark field microscopy**

**FIG.A.3:**

**Leptospira more prevalent during rainy season**

**FIG.A.4:**

**Leptospira survival more in stagnant water and common in slum people**

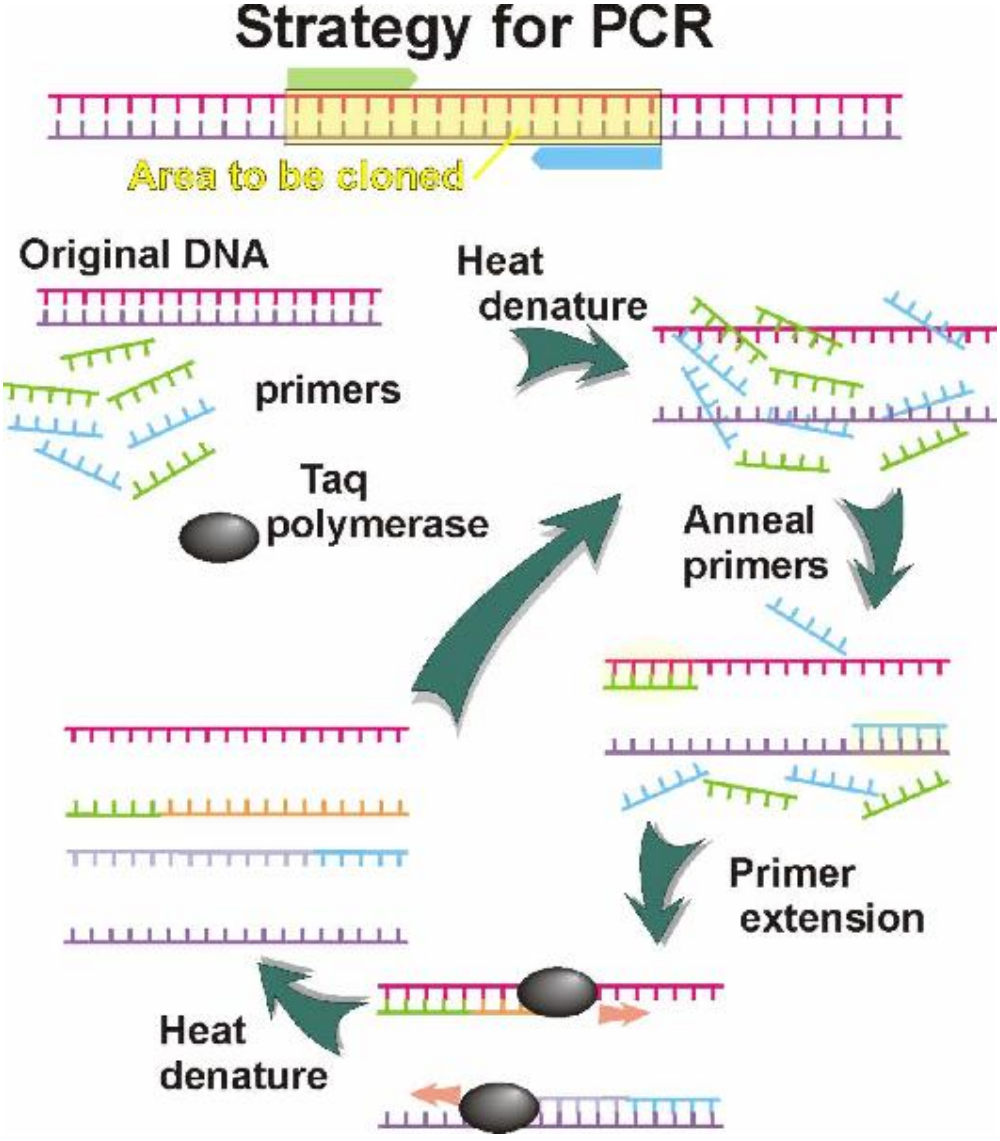



**FIG.A.5: Principle of PCR**

**FIG. A.6: Dark field Microscope**

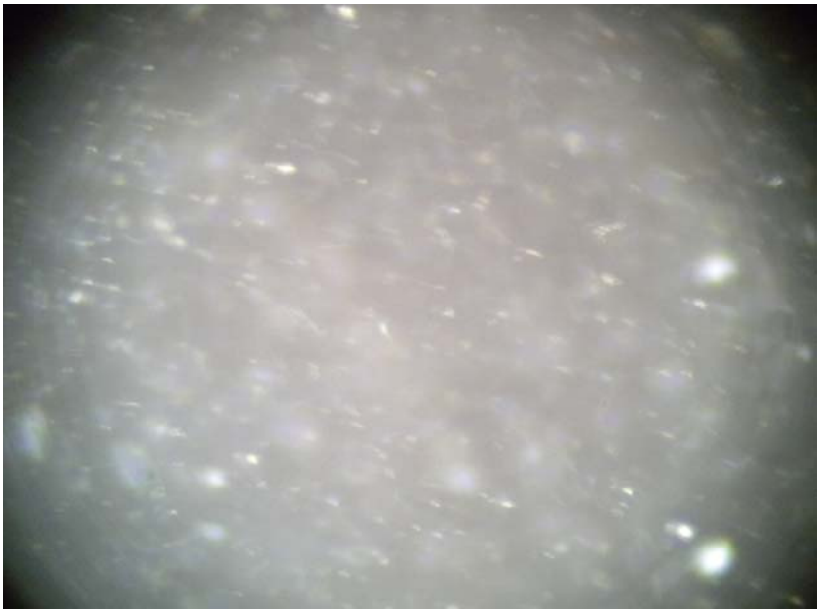


**FIG.A.7: Elongated live leptospires under DFM**

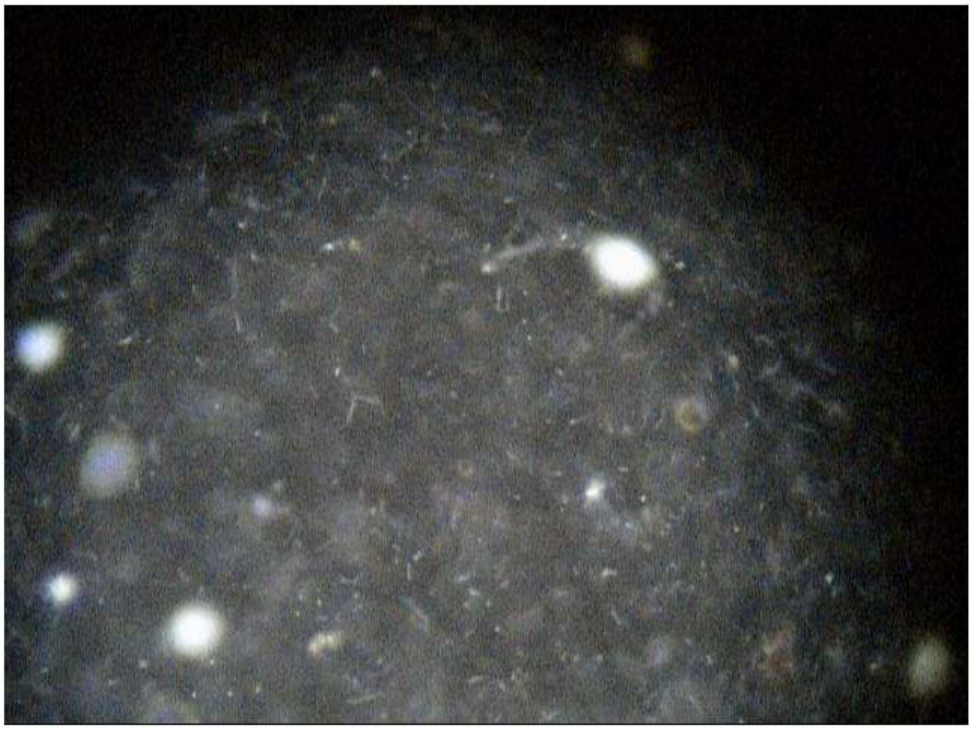


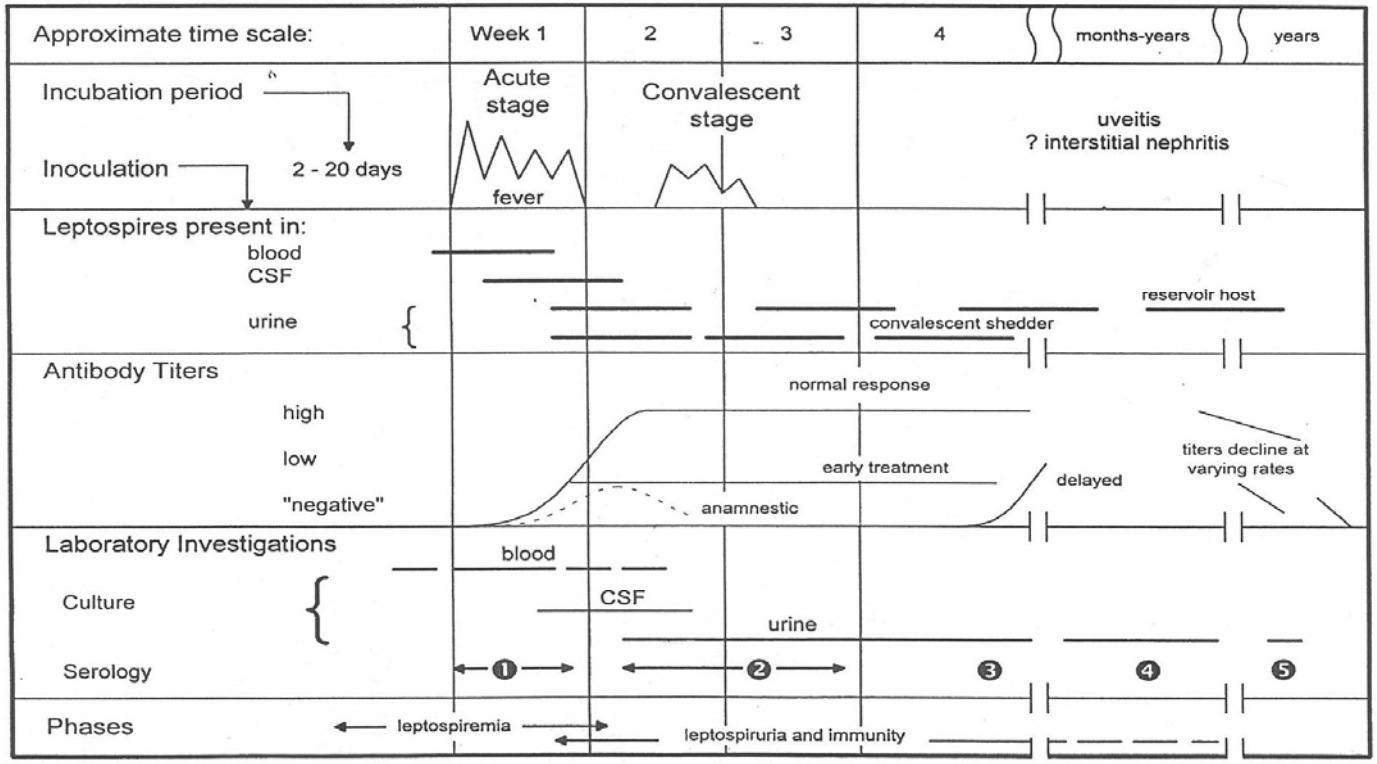
**FIG. A.8: MAT**


**FIG. A.9: Thermal Cycler**

**FIG. A.10 : Loading the DNA samples on 2% Agarose gel electrophoresis**


**FIG. A.11 : Showing DNA Samples run in 2% agarose gel.**
**1 : Positive Control**
**2 – 5 : Samples**


**FIG. A.12 : Showing DNA Samples run in 2% agarose gel.**
**1 : DNA Ladder 1KB**
**2 : Positive Control**
**3-23 : Samples**




**Biphasic nature of leptospirosis and relevant investigations at different stages of disease.** Specimens 1 and 2 for serology are acute-phase specimens, 3 is a convalescent-phase sample which may facilitate detection of a delayed immune response, and 4 and 5 are follow-up samples which can provide epidemiological information, such as the presumptive infecting serogroup. [14]

## Summary of Laboratory Diagonosis of Leptospirosis[23]

| Test | Advantages | Disadvantages | Correlation of Clinical Disease with Investigations at different stages of disease | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Sample | 1st week | 2nd week | 3rd week | 4th week | Month-years | Years |
| A. Microscopy: | | | | | | | | | |
| ·Dark Field (DFM) | Convenient for visualizing leptospires in blood, urine, rarely in CSF | DFM-Lacks sensitivity and specificity, approximately $10^4$ leptospires/ml are necessary for one cell per field to be visible under DFM | Blood | + | - | - | - | - | - |
| ·Immunoflorescence | | | CSF | +/- | +/- | - | - | - | - |
| ·Light | | | Dialysate Fluid | +/- | +/- | - | - | - | - |
| ·Immunoperoxidase staining | | | Urine:Convalescent shedder | - | + | + | + | +/- | +/- |
| ·Silver staining | Demonstration of leptospires in tissues | | Reservoir host | - | + | + | + | + | + |
| ·Warthin-Starry stain | | | | | | | | | |
| ·Immunohistochemistry | | | | | | | | | |
| ·Quantitative buffy coat | | | | | | | | | |
| B. Culture: | Gives confirmed diagnosis | Cumbersome | | | | | | | |
| C. Serology: | | | | | | | | | |
| a) Antibody Detection | | | | | | | | | |
| Paired sera required to confirm diagnosis. Fourfold or greater rise in titer between paired sera confirms diagnosis | Positive from 2nd week of illness onwards | | Normal Response | - | +/- (high titres) | + (high titres) | + (high titres) | +/- (declining titres) | +/- (declining titres) |
| -Serogroup Specific Tests | | | | | | | | | |
| ·Microscopic Agglutination Test (MAT) | Gold Standard, High sensitivity, Detection of group-specific antibodies possible | Complex due to requirement of maintaining strains for the preparation of live antigens | Early Treatment | - | +/- (low titres) | + (low titres) | + (low titres) | +/- (declining titres) | +/- (declining titres) |
| | | | Delayed Response | - | - | - | +/- | + | +/- (declining titres) |
| -Genus Specific Tests | | | | | | | | | |
| ·IgM ELISA | Most widely used laboratory method for leptospira diagnosis, Time taken to perform assay varies from 30 seconds to 4 hours | | Anamnestic Response | -/+ | +/- | - | - | - | - |
| ·IgG ELISA | | | | | | | | | |
| ·Indirect Fluorescent Antibody Tests (IFAT) | | | | | | | | | |
| ·IgM Dipstick | | | | | | | | | |
| ·Macroscopic Slide Agglutination | | | | | | | | | |
| ·Lateral Flow Assay | | | | | | | | | |
| ·Indirect hemagglutination assay | | | | | | | | | |
| ·Microcapsule agglutination | | | | | | | | | |
| ·Counterimmunoelectrophoresis | | | | | | | | | |
| ·Complement Fixation | | | | | | | | | |
| b) Antigen Detection | | | | | | | | | |
| ·Radioimmunoassay (RIA) could | Greater specificity than dark-field microscopy, Can detect upto $10^4$ to $10^5$ leptospires/ml | | Blood | + | - | - | - | - | - |
| ·Enzyme-linked immunosorbent assay (ELISA) | | | CSF | +/- | +/- | - | - | - | - |
| ·Chemiluminescent immunoassay | | | Dialysate Fluid | +/- | +/- | - | - | - | - |
| ·Staphylococcal coagglutination | | | Urine:Convalescent shedder | - | + | + | + | +/- | - |
| | | | Reservoir host | - | + | + | + | + | + |
| D. Molecular diagnosis: | | | | | | | | | |
| ·Dot-blotting | | | | | | | | | |
| ·In situ hybridization | | | | | | | | | |
| ·PCR | Successful method to detect *Leptospira* DNA in serum and even better in urine in the first week of infection, also used to detect *Leptospira* DNA in tissues for post-mortem diagnosis, which | Sensitivity lower than PCR,not extensively used for diagnosis<br><br>Inability of most PCR assays to identify the | | | | | | | |

**Table 1: Agewise Distribution of Voluntary Blood Donors**

| Sl. No. | Age | No. of Donors |
|---|---|---|
| 1. | 18-20 Years | 10 |
| 2. | 21-30 Years | 45 |
| 3. | 31-40 Years | 18 |
| 4. | 41-50 Years | 17 |
| 5. | 51-60 Years | 10 |
| **TOTAL** | | **100** |

| | | | | | |
|---|---|---|---|---|---|
| Mean | - | 32.54 | Minimum Age | - | 19 Yrs |
| Standard Deviation | - | 11.02 | Maximum Age | - | 56 Yrs |
| Range | - | 37 | | | |

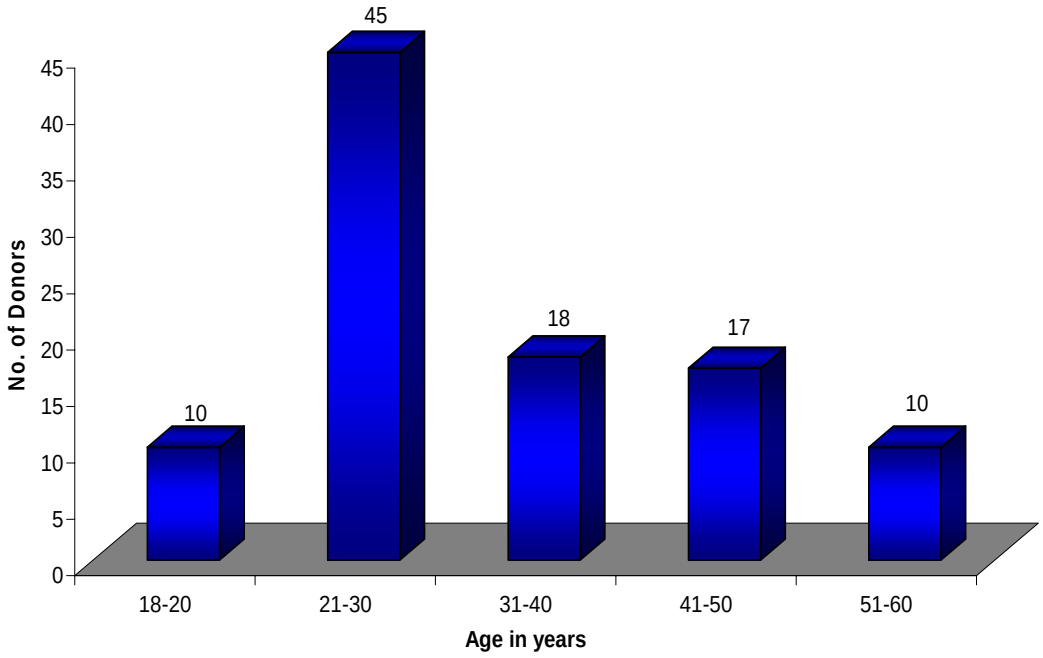



**Fig.1.B:**

**Table 2: Sex Distribution of the Voluntary Blood Donors**

| Sl. No. | Sex | No. of Donors |
|---|---|---|
| 1. | Males | 75 |
| 2. | Females | 25 |
| | **Total** | **100** |

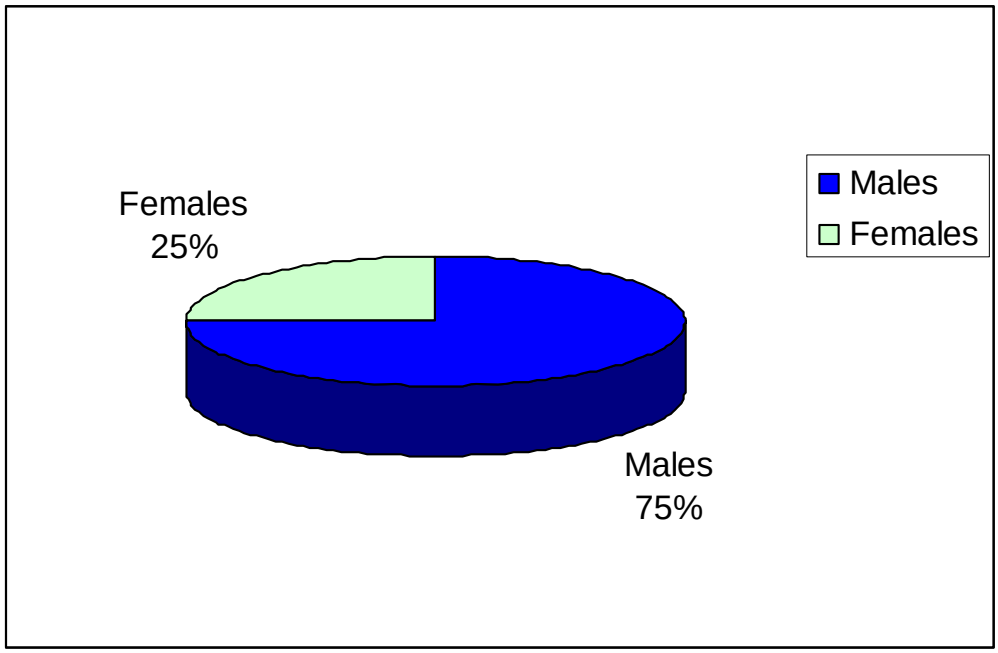



**Fig.2.B**

**Table 3: Occupation Distribution of Voluntary Blood Donors**

| Sl. No. | Occupation | No. of Donors |
|---|---|---|
| 1. | Student | 16 |
| 2. | Professional | 43 |
| 3. | Clerical | 26 |
| 4. | Coolie | 10 |
| 5. | Unemployed | 5 |
| | **TOTAL** | **100** |

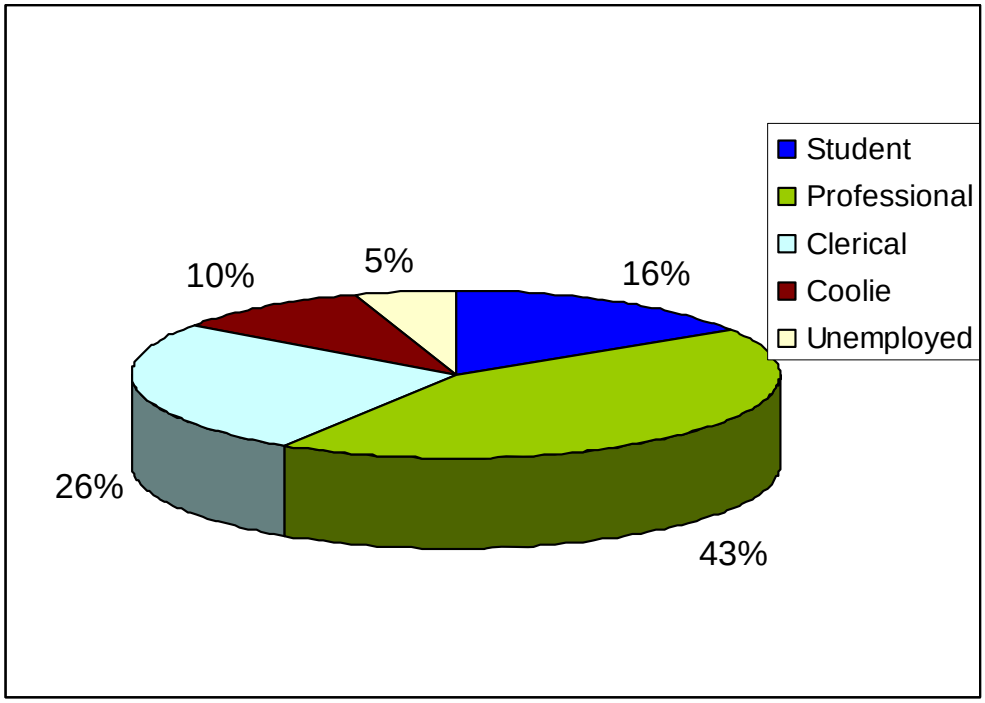



**Fig.3.B**

**Table 4: Zonal Distribution of Voluntary Blood Donors**

| Sl. No. | Chennai Zones | No. | Percentage |
|---|---|---|---|
| 1. | 1 | 3 | 3% |
| 2. | 2 | 4 | 4% |
| 3. | 3 | 4 | 4% |
| 4. | 4 | 7 | 7% |
| 5. | 5 | 7 | 7% |
| 6. | 6 | 2 | 2% |
| 7. | 7 | 9 | 9% |
| 8. | 8 | 7 | 7% |
| 9. | 9 | 11 | 11% |
| 10. | 10 | 20 | 20% |
| 11. | Outside Chennai | 26 | 26% |

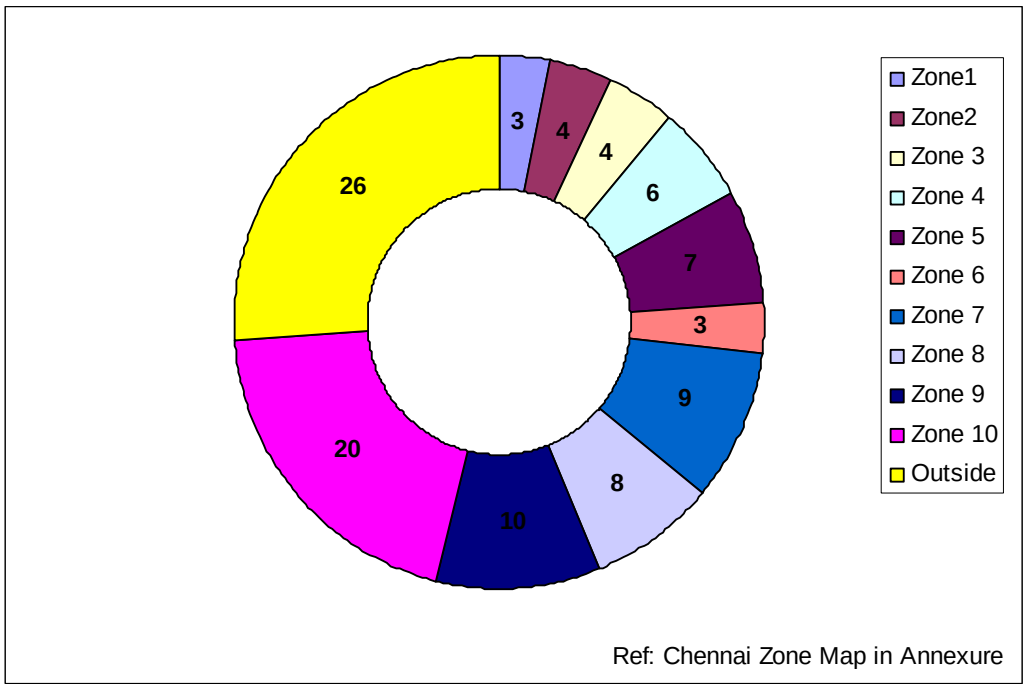



**Fig.4.B**

**Table 5: Laboratory Tests**

Comparison of MAT & DFM

| LABORATORY TESTS | | |
|---|---|---|
| **DFM** | **MAT** | **TOTAL** |
| + | + | 21 |
| + | - | 10 |
| - | + | 19 |

**Table 6: Correlation between MAT and DFM**

| | | DFM | | Total | P Value |
|---|---|---|---|---|---|
| | | **+ve** | **-ve** | | |
| MAT | +ve | 21<br>(52.5%)<br>[67.7%] | 19<br>(47.5%)<br>[27.5%] | 40 | P<0.0015** |
| | -ve | 10<br>(16.7%)<br>[32.3%] | 50<br>(93.3%)<br>[72.5%] | 60 | |

**Note:**

** - denotes significance at 1% level

* - denotes significance at 5% level

The value within ( ) refers to row percentage

The value within [ ] refers to column percentage

**Table 7: Serovars in MAT alone Positive (n=40)**

| Sl. No. | Serovars | No. (%) (n=40) | P Value |
|---|---|---|---|
| 1. | Autumnalis | 14(35%) | P<0.017* |
| 2. | Australis | 10(25%) | |
| 3. | Patoc | 10(25%) | |
| 4. | Icterohaemorrhagiae | 2(5%) | |
| 5. | Canicola | 4(10%) | |

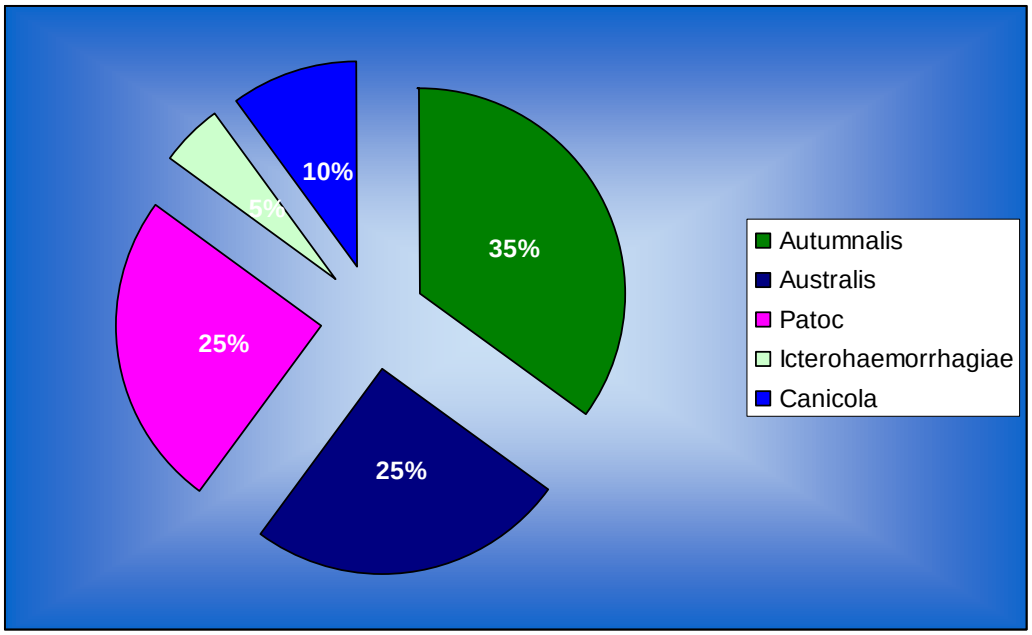



**Fig.5B**

**Table 8: Prevalence of serovars in both DFM & MAT Positive Donors (n=21)**

| Sl. No. | Serovars | No. (%) | P value |
|---|---|---|---|
| 1. | Autumnalis | 8(38%) | $0.143^{NS}$ |
| 2. | Australis | 6(29%) | |
| 3. | Canicola | 2(10%) | |
| 4. | Patoc | 3(14%) | |
| 5. | Icterohaemorrhagiae | 2(10%) | |
| | **TOTAL** | **21(100% )** | |

Note: NS – Non Significant.

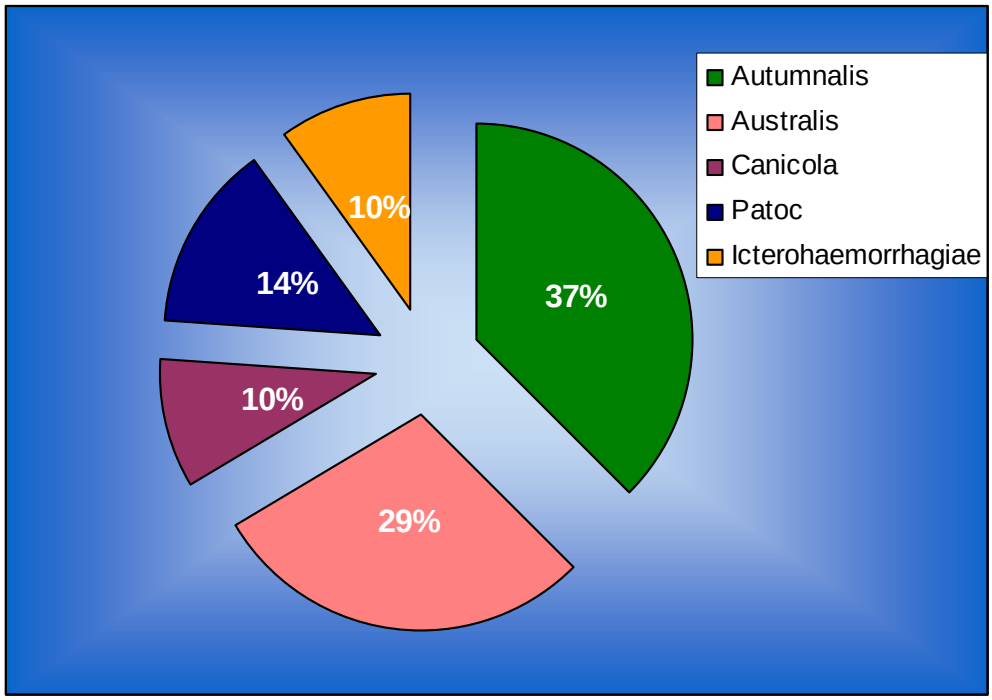



**Fig.6.B**

**Table 9: Prevalence of serovars in DFM Negative & MAT Positive Donors (n=19)**

| Sl. No. | Serovars | No. (%) |
|---|---|---|
| 1. | Autumnalis | 6 (32%) |
| 2. | Australis | 4(21%) |
| 3. | Canicola | 2(11%) |
| 4. | Patoc | 7(37%) |
| | **TOTAL** | **19(100%)** |

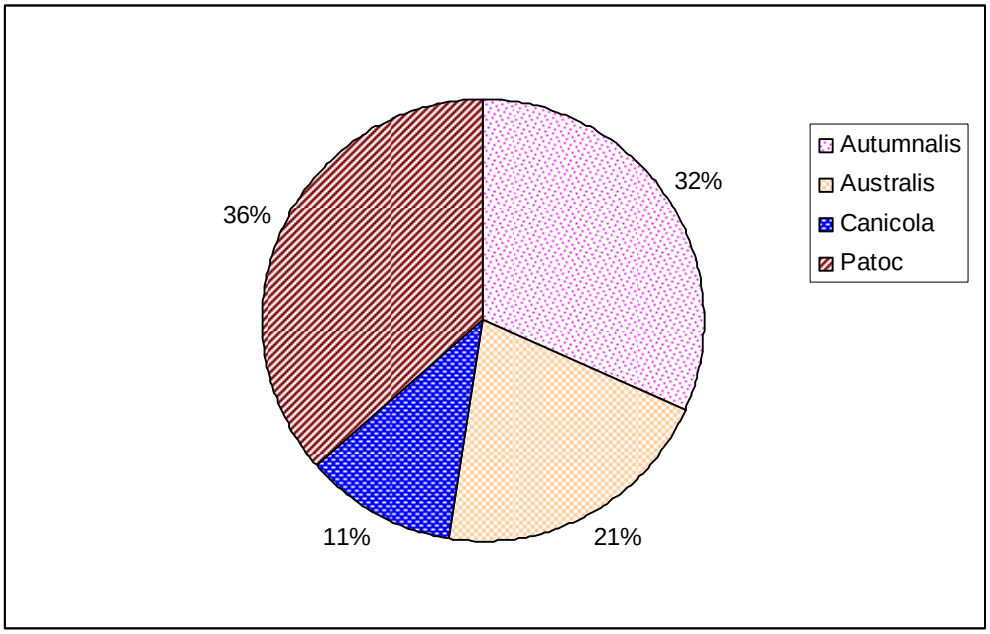



**Fig.7.B**

**Table 10: Association of Leptospirosis with other TTIs**

| | | |
|---|---|---|
| HBsAg Positive | - | 2 |
| HBsAg Negative | - | 98 |
| **TOTAL** | **-** | **100** |

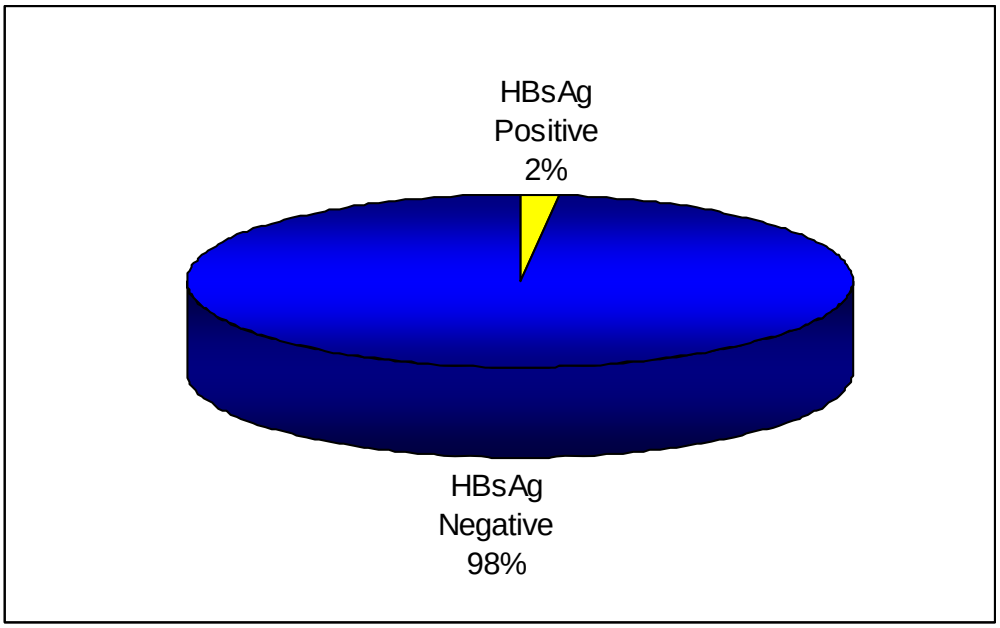



**Fig.8.B**

**Table 11: Age groupwise distribution of DFM +ve**

| Sl. No. | Age in Years | DFM +ve (n=31) | P Value |
|---|---|---|---|
| 1. | 18 – 20 (n=10) | 2(6.5%) | P<0.032* |
| 2. | 21 – 30 (n=45) | 9(29%) | |
| 3. | 31 – 40 (n=18) | 9(29%) | |
| 4. | 41 – 50 (n=17) | 9(29%) | |
| 5. | 51 – 60 (n=10) | 2 (6.5%) | |

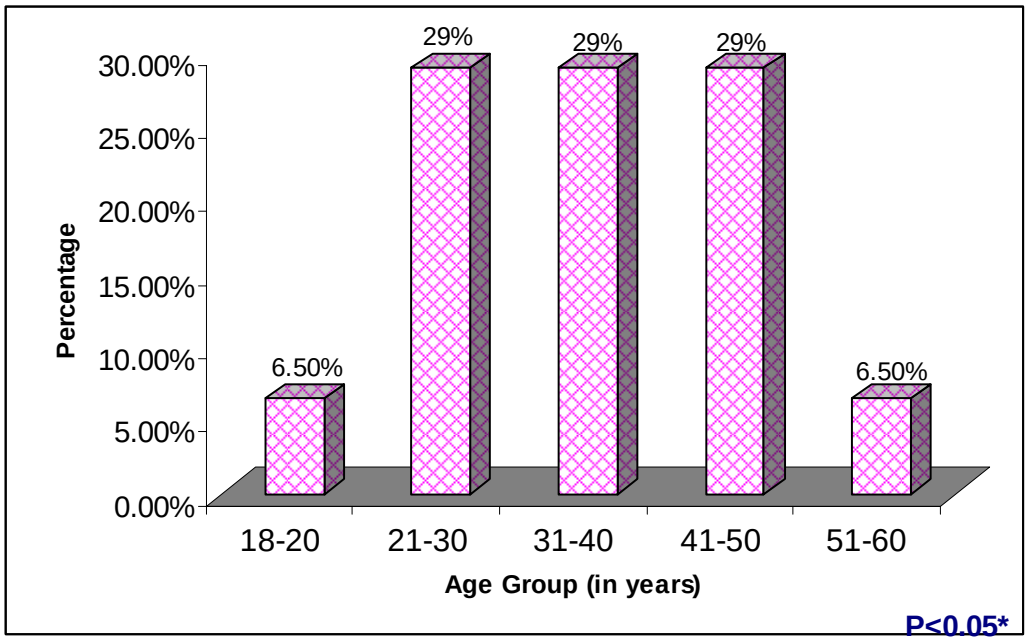



**Fig.9.B**

**Table 12: Age groupwise distribution of MAT +ve**

| Sl. No. | Age in Years | MAT +ve (n=40) | P Value |
|---|---|---|---|
| 1. | 18 – 20 (n=10) | 2(2.5%) | P<0.00057** |
| 2. | 21 – 30 (n=45) | 9(32.5%) | |
| 3. | 31 – 40 (n=18) | 9(30%) | |
| 4. | 41 – 50 (n=17) | 9(30%) | |
| 5. | 51 – 60 (n=10) | 2 (5%) | |

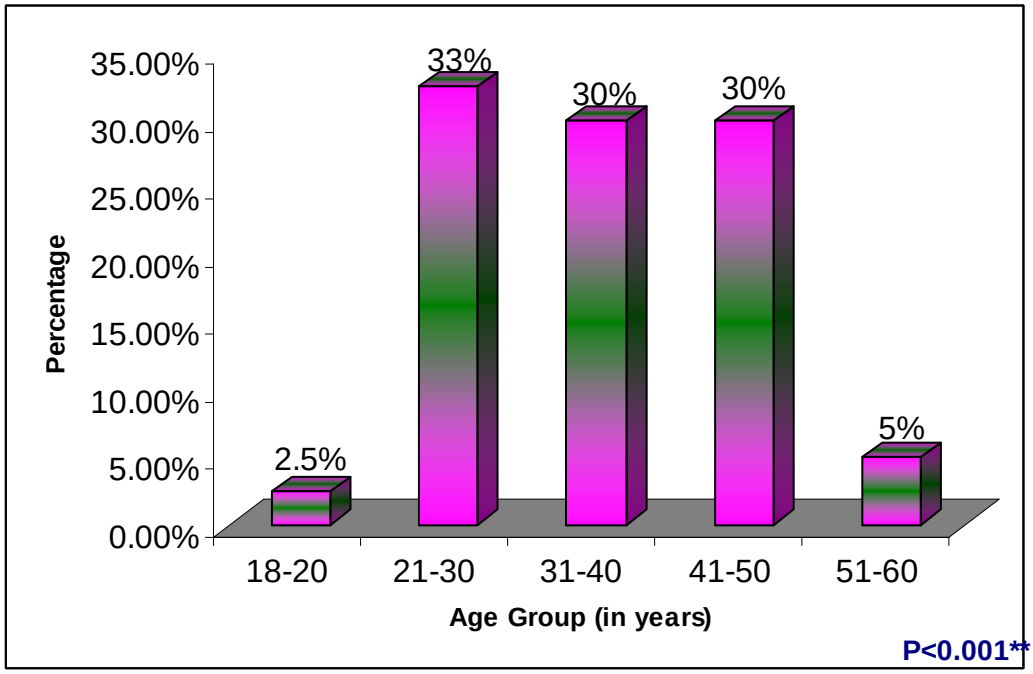



**Fig.10.B**

**Table 13: Occupationwise distribution of MAT Positive donors**

| Sl. No. | Occupation | Percentage | P Value |
|---|---|---|---|
| 1. | Professional (n=43) | 45% | P<0.0000** |
| 2. | Clerical (n=26) | 33.5% | |
| 3. | Student (n=16) | 5% | |
| 4. | Coolie (n=10) | 15% | |
| 5. | Unemployed (n=5) | 3% | |

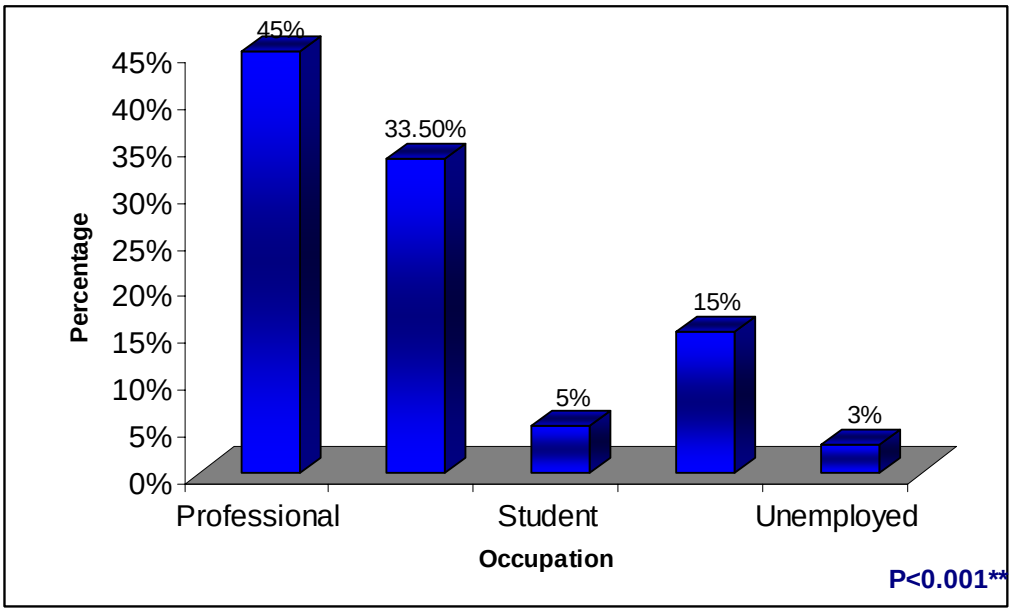



**Fig.11.B**

**Table 14: Zonalwise Distribution of MAT Positive Donors**

| Sl. No. | Chennai Zones | Percentage |
|---|---|---|
| 1. | 1 | 3% |
| 2. | 2 | 3% |
| 3. | 3 | 5% |
| 4. | 4 | 5% |
| 5. | 5 | 9% |
| 6. | 6 | 3% |
| 7. | 7 | 10% |
| 8. | 8 | 13% |
| 9. | 9 | 10% |
| 10. | 10 | 20% |
| 11. | Outside Chennai | 23% |

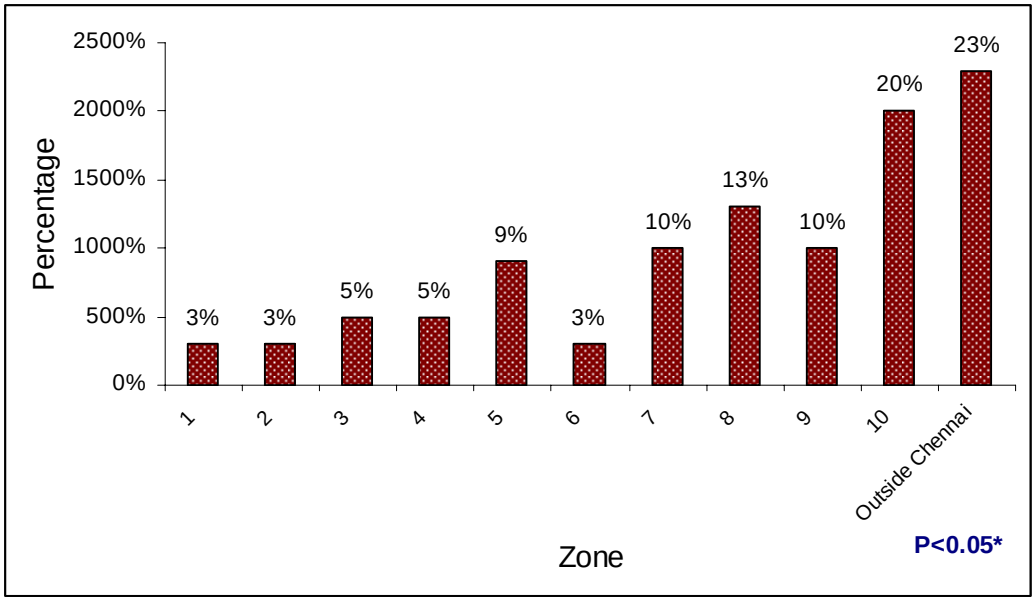



**Fig.12.B**

# CHENNAI CORPORATION CITY – ZONE MAP