

**STUDY OF SEROPREVALENCE OF LEPTOSPIROSIS USING
CONVENTIONAL AND MODERN DIAGNOSTIC TOOLS**

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UNIVERSITY OF MUMBAI**

**FOR THE DEGREE OF
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IN
APPLIED BIOLOGY**

**SUBMITTED BY
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**UNDER THE GUIDANCE OF
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**HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
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Study of Seroprevalence of Leptospirosis Using Conventional And Modern Diagnostic Tools

A Thesis Submitted to the
University of Mumbai

For the
**Ph. D. (Science) degree,
in Applied Biology**

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STATEMENT BY THE CANDIDATE

As required by the University Ordinance No. 770, I wish to state that the work embodied in this thesis titled “ **Study of seroprevalence of leptospirosis using conventional and modern diagnostic tools** ” forms my own contribution to the research work carried out under the guidance of

Dr. (Mrs.) Ranjana A. Deshmukh at the Haffkine Institute for Training, Research and Testing, Mumbai – 12. This work has not been submitted for any other degree of this or any University. Whenever reference has been made to previous works of others, it has been clearly indicated as such and included in the Bibliography.

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Dr. (Mrs.) R. A. Deshmukh
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Dedicated to

Shri Sidhhivinayak

&

Guru Shri Kadsidhheshwar

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Introduction

1 INTRODUCTION



“The power of water has been highlighted over the past year, following the Boxing tsunami and the devastation wreaked on New Orleans by hurricane Katrina. How water can represent a danger in other ways; for example, in the transmissic leptospirosis” - Richard A Collins.[1]

Leptospirosis has been recognized as an important emerging global public health problem because of its epidemic proportions and increasing incidence in both developing and developed countries.[2] Leptospirosis, the infection caused by leptospires, is the most widespread zoonosis in the world. Leptospires are ubiquitous spirochetes. While saprophytic species is a usual contaminant of surface waters, the primary habitat of pathogenic species is the distal tubules of kidneys in rodents, the reservoir hosts of various other animal species. Pathogenic leptospires shed in the urine of carrier animals contaminate the environment and cause human and animal infection throughout the world.[3] It has wide geographical distribution and occurs in tropical, subtropical and temperate zones and may be prevalent in both urban and rural settings. Annual incidence is estimated from 0.1–1 per 100000 in temperate climates to 10–100 per 100000 in the tropics. A disease incidence of more than 100 per 100000 is encountered during outbreaks in high risk groups. Worldwide prevalence rates remain underestimated, likely as a result of the protean nature of the disease.[4] The International Leptospirosis Society (ILS) made an attempt to compile data on occurrence of leptospirosis in various countries. The data shows that on an average 10000 severe cases requiring hospitalization occur annually all over the world. Vast majority of leptospiral infections are caused by

In a small proportion of patients, severe complications may set in. In such cases, would be clinical manifestations of multiple organ involvement and the case fatality could be more than 40%. Because of the protean manifestations of leptospirosis it is misdiagnosed and under-reported. In endemic areas, leptospirosis is a major cause of various clinical syndromes such as jaundice, renal failure, myocarditis and at pneumonia. Some countries such as Thailand, where leptospirosis is under surveillance have recorded an increase in the incidence of leptospirosis. The annual incidence of leptospirosis increased from 0.3/100000 persons during the period 1982–1983 to 3.3/100000 persons in 1997–98. Most countries in the South East Asia region are endemic to leptospirosis. A multi-centric study in India showed that leptospirosis accounts for about 12.7% of cases of acute febrile illness attending hospitals.[5] On global data collected by the International Leptospirosis Society (ILS) surveys the number of currently 350000 to 500000 severe cases of leptospirosis annually. However, this is probably a heavy underestimation and the true extent of leptospirosis remains unknown because worldwide surveillance is lacking.

Previous Outbreaks of Leptospirosis

Outbreaks usually occur on the aftermath of natural calamities such as cyclones, floods and flash-floods. Large number of outbreaks has been reported from several countries in recent times.

Since the original identification of leptospirosis by Dr. Weil,[6] outbreaks of leptospirosis have occurred sporadically throughout world. Several epidemics of leptospirosis have been reported worldwide during the past century, whereas in India, leptospirosis is endemic in most of the urban areas in Southern and Western India, where outbreaks

Western parts of India since the first case of leptospirosis in India was reported by [5] and Goyal in 1929.[6] The original outbreak in 1929 occurred among bund construction workers in a village on South Andaman.[7] Since the 1980s, there have been increasing numbers of leptospiral cases reported in the states of Orissa, Maharashtra, Karnataka, Tamil Nadu and Kerala. Leptospirosis outbreaks frequently occur during tin mining, flooding and natural disasters. Notable global outbreaks include: [8] 186 U.S. troops in the Panama Canal zone in 1961 who had been in a jungle exercise 10–15 days earlier, (2) Italy in 1984 due to a contaminated water fountain, (3) Thailand from 1987 through 2000, (4) Nicaragua in 1995, [9] Peru and Ecuador during heavy flood in the spring of 1998, and (6) 110 cases in triathletes (from swimming in contaminated Spring field) in June and July of 1998 in Illinois and Wisconsin in the United States [12] During the Eco-Challenge-Sabah 2000 multisport expedition race in Borneo, Malaysia, several participants from multiple countries were infected with leptospirosis. The diagnosis was based on epidemiological evidence and detected with ELISA and MAT.[13]

Following the Mumbai flood in July 2000, a study of 53 children <12 years of age admitted to a pediatrics department were tested for leptospirosis. Serological investigation indicated that almost one-third of the children had acute leptospirosis.[14]

In 2003, there were at least two serious outbreaks of leptospirosis in India. In Kerala, 100 individuals had developed leptospirosis and as many as 115 persons had died from it. In September of 2003, 24 people died from leptospirosis in South Gujarat within 1 month. These deaths were from the 177 individuals infected with leptospirosis from 131 villages. and, in fact, there had been unconfirmed reports that over 500 people were ill.[15]

cases of leptospirosis and at least 75 deaths in South Gujarat.[18]

Elsewhere in the world in 2004, a professor from the University of Hawaii and a graduate student were infected with leptospirosis while cleaning out his laboratory that had been flooded by an overflowing stream.[19]

In 2005, after heavy flooding in Maharashtra state and specifically in Mumbai region there were at least 100 deaths from leptospirosis within a two-day period.[20]

Elsewhere in the world in 2005, there were at least 27 cases and 8 deaths of leptospirosis in the Ukraine. Concurrent with these human cases, a significant percentage of the local mice populations, up to 25%, were testing positive for leptospirosis.[21] In the Dominican Republic at the end of 2005, there were 4 deaths due to leptospirosis. These cases were attributed to problems with sanitation and problems connected with municipal garbage collection.[22] In addition, there were at least 6 deaths reported from leptospirosis in Guyana at the end of 2005. They did not find any particular pattern in the cases; and, the first death had actually occurred prior to the start of heavy rain and flooding in the country.[23]

In April of 2006, there was a rare outbreak of leptospirosis in Tajikistan. There had not been an outbreak of leptospirosis in the country in more than a decade. At least 15 cases of leptospirosis occurred; and, these were attributed to recent work on the public water supply system.[24] Further, in October of 2006, two clinically diagnosed cases of leptospirosis were detected in the United Kingdom. The two individuals had been on a fishing holiday in the Picardie region of Northern France.[25]

In 2007, there were nearly 400 cases and at least 5 deaths attributable to leptospirosis in the Santa Fe province of Argentina. This outbreak followed the severe storm that

Sri Lanka in 2007. There were also at least 3 cases of leptospirosis in the Ukraine. cases were attributed to an increase in rats.[27-29]

In January of 2008, there were concerns about a leptospirosis outbreak in Jamaica high school students were hospitalized with symptoms of leptospirosis.[30] There also been recent outbreaks of leptospirosis in New Caledonia. In fact, New Caledonia is one of the highest rates of leptospirosis in the Pacific region; and leptospirosis is more common than Tuberculosis, Salmonellosis, Hepatitis A, Hepatitis B, and Gonorrhea in New Caledonia.[31]

Also, in 2008, a new leptospiral species, *Leptospira licerasiae*, was discovered in the Peruvian Amazon. It may be responsible for up to 40% of the cases in the area and is an important cause of leptospirosis in the Peruvian Amazon region of Iquitos. In a study testing of 881 patients with fever in the region, 41% had antibodies that only reacted to this new bacterium.[32] This leptospiral species is unique at the genomic level and has novel biological characteristics. Based on phylogenetic analysis, this species is considered part of the intermediate clade (between the pathogenic and nonpathogenic *Leptospira* clades); however, it does share some characteristics with the pathogenic serovars. For example, this new species is sensitive to 8-azaguanine, a test which is used to differentiate pathogenic and non-pathogenic *leptospira*.[33]

Morbidity and Mortality

Leptospirosis has not been a notifiable disease in the U.S. since 1994, and the current incidence in humans is not known with certainty. From 1987 to 1993, 43–93 cases were reported annually. Currently, the U.S. Centers for Disease Control and Prevention (CDC)

thought to be under diagnosed and underreported since many cases are asymptomatic and self-limiting. The incidence of infection is seasonal, with most seen during summer and fall in temperate regions. In tropical climates, the peak incidence occurs during the rainy season. Occupational exposure is thought to be responsible for 30–50% of cases. Occupations with a high risk of infection include sewer workers, miners, plumbers, farm workers, veterinarians, pet shop owners, abattoir workers, handlers, slaughterhouse workers, workers in the fishing industry and the military. It is estimated that 8–29% of those who work with livestock have antibodies to *Leptospira*. [34] Recreational activities that increase the risk of leptospirosis include gardening and water sports such as canoeing, swimming and white-water rafting. Residents of some urban areas are exposed via rat urine. Most cases of leptospirosis are asymptomatic or mild. The overall case fatality rate is 1–5%. The mortality rate increases with the form, and is higher in the elderly. The anicteric form is rarely fatal. The icteric form, which occurs in 5–10% of all patients, has an overall mortality rate of 5–15%. The 54% case fatality rate in severe cases with myocardial involvement. [34]

Leptospirosis is primarily a monsoon disease, which affects the domestic and wild animals as well as humans. It was not long after their discovery of leptospires as the cause of Weil's disease in Japan that the same Japanese group described the role of animals as carriers. [35-37] This important observation opened the way for understanding the epidemiological principles of transmission by carrier animal vectors, and control, and paved the way for future research on other potential animal reservoirs of leptospires. It took several more years before other domesticated or wild animal sources were recognized and the widespread zoonotic implications of leptospirosis were understood. [37] Rats are the most common source of infection for humans and act as a reservoir host. Rural farm workers

rodents indicated 4 species i.e., Norway rat (*Rattus norvegicus*), house rats (*rattus*), lesser bandicoots (*Bandicoota bengalensis*) and larger bandicoot (*Band indica*) as associated with the disease. Since 14 rodent species are predominant in and leptospiral infection spreading in this part of the world, there is an immediate for screening the remaining species for their role as vector for leptospirosis.[38] R are often incriminated as the source of infection to human beings. Although s icterohaemorrhagiae has been often associated with rodents, other serovars have been isolated. In India, the natural leptospiral infection in rat was first documented Kolkata, subsequently in Mumbai and in Kerala with the isolation of autumn hebbomadis and javanica in rats and bandicoots. Serovar javanica was isolated Karnataka and Tamil Nadu. In Madurai, 46% positivity of viable leptospires belonging the serogroups Autumnalis and Javanica in field rats has been reported.[39] R bandicoots have shown evidence of anti-leptospiral antibodies following isolation leptospires from suspected human patients from the suburbs of Chennai. Elsewhere Tamil Nadu anti-leptospiral antibodies (52.1%) were evident from the field rats. While relatively low seroprevalence was observed among the rat population, yielding isolates from the Andaman and Nicobar archipelago.[5] Very high seroprevalence and carrier rates (90.9% and 82.9% respectively) were observed among house rats Terceira Island in Azores. In one of the study at Republic of Croatia, small rodent seen to be serologically positive, especially the species *Mus musculus*.[40]

Leptospirosis in domesticated animals was identified, at first as a disease primarily dogs. The potential for infection of humans was recognized. Both the canicola and

sensu lato, of which eight are most important to dogs and cats. The disease, which was caused by serovars most commonly incriminated (*icterohaemorrhagiae* and *canicola*) with cases of “classic” canine leptospirosis, has decreased greatly in prevalence with the development of commercial vaccines containing antigens from these serovars. However, reported clinical cases associated with other pathogenic serovars (particularly *pomona*, *grippityphosa*, and *bratislava*) not included in these vaccines have seen a dramatic increase over the past 10 to 15 years. Serovars *pomona* and *grippityphosa* have been associated with numerous cases of acute renal failure, and serovars *bratislava*, *hardjovis*, and *bataviae* have been implicated in both hepatic and renal disease as well.[41]

Leptospirosis in dogs is known as Stuttgart disease. In a sero-survey conducted in 1992, 245 kennelled dogs in Italy, 72 were found to be seropositive. Venkataraman and Nedunchelliyan (1992) reported an outbreak of leptospirosis in human beings and dogs in Chennai City. Following the outbreak, a serosurvey was conducted among humans and dogs. Seroprevalence was 50.5% among humans and 21.3% in dogs. *Leptospira* belonging to serovar *icterohaemorrhagiae* were isolated from a human patient and *canicola* from a dog.[5]

The prevalence of *Leptospira* antibodies in dogs has been reported among different countries: 21.3% in India (Venkataraman and Nedunchelliyan, 1992), 6.36% in Italy (Cerri et al., 2003) while the prevalence was 11% in Thailand (Tor Meeyam, 2004).[42]

Bovine leptospirosis is an economically significant disease and occurs throughout the world. The disease results in abortions, stillbirths, loss of milk production, and zoonosis.[43]

Leptospiral infection among cattle was first recorded in Russia. Cattle throughout the world over may be infected with serovars *hardjovis*, *pomona*, and *grippityphosa*.

762 dairy cattle belonging to 81 herds in Spain was carried out using Micro Agglutination Test (MAT) with 11 leptospiral serovars as antigens. Lept antibodies were detected in 8% of the cattle. A sero-survey among animal population in Andaman and Nicobar Islands showed that about 40% of the cows and 26% of the goats were seropositive. A study on 40 cows in a village near Chennai following an outbreak of leptospirosis in cattle showed antibodies against leptospire in 68% of the cows. (Rajasekhar et al. 1983)

Pigs are commonly infected with serovars pomona, tarassovi, grippotyphosa, bratsejroe, icterohaemorrhagiae and canicola (Faine 1994). Adult non-pregnant infected pigs are usually symptom-free and become chronic carriers. The disease in pigs is usually subclinical (no apparent signs of illness) except for abortions, which usually occur during the last two to three weeks of pregnancy. Piglets may be born weak and die shortly after birth.[44]

In India, the disease has been recorded in Cattle, Buffalos, Horses, Sheep, Goats and Dogs (Arora, 1977; Uppal and Singh 1982). Among the earliest reports of leptospirosis that have been recorded were in Chennai in 1932 by Ayyar; Sawney and Saxena (1933) from Madhya Pradesh; Rajasekhar and Nanjiah (1971) from Mysore; Tripathi (1972) from Orissa; Verma (1982) from Punjab and Dwivedi (1988) from Uttar Pradesh. Seroprevalence of leptospirosis in animals using sera collected from various states in India during a period of 10 years beginning 1990 was carried out. In this study a total of 271 goat, 551 sheep, 756 equine, 204 dog and 166 swine sera received from various states were tested and 14.3%, 15.2%, 9.9% and 9.0%, respectively, were positive.[45]

Occupational and recreational exposures account for 30–50% of leptospirosis. Typically, individuals who work outdoors or with animals are at risk of the disease. Previously, most cases were due to occupational exposure. However, more cases are now occurring due to recreational exposure; although, occupational exposure is still a significant concern. Prime examples of high-risk occupational groups include pet owners, veterinarians, veterinary technicians, agricultural workers, abattoir workers, plumbers, meat handlers, meat inspectors, hunters, laboratory staff, butchers, heavy meat carriers, coal miners, construction workers, fish industry workers, military personnel, civil emergency personnel, sewer workers, and garbage collectors.[10, 11, 46-48]

Leptospirosis has been known by a variety of names that reflect the method (usually occupational) by which the disease was contracted. Alternative names for leptospirosis include rice field fever (in Indonesia), seven-day fever (in Japan), cane cutter's disease (in Australia), swineherd's disease, dairy farm fever, swamp fever, Fort Bragg fever (in the United States), and mud fever.[12, 19] As for recreational exposures, high-risk recreational exposures for leptospirosis include adventure traveling to the tropics, explorers, camping, hiking and riding trail-bikes through contaminated pools, swimming, canoeing and kayaking, fishing, water skiing and windsurfing, and outdoor sports played in potentially infected water.[19] In addition, urban dwellers are also at increased risk because of potential exposure to infected rats. Also, children are at risk by playing in contaminated water or having contact with infected animals.[19] Epidemiological studies indicate that infection is commonly associated with

hiking, picnicking, swimming and canoeing.[49] A study conducted on risk groups in India showed that in a veterinary group, out of 42 patients, 15 (35.71%) showed evidence of leptospirosis of which 10 were livestock farm workers, 2 were veterinarians and attendants and 1 laboratory technician.[50] An outbreak of leptospirosis in the summer of 1999 associated with recreational exposure was reported in the Yaeyama Islands, Japan. [51] Study in Salem, South India showed 68.3% sero-positivity among rice mill workers using MAT.[52] There has been also report of outbreak of leptospirosis in travelers returning from participation in the Eco-Challenge Sabah 2000.[53] Keeney et al. identified leptospirosis as the cause of an outbreak of acute undifferentiated febrile illness that occurred among military recruits participating in training exercises in the high jungle region of Peru.[54]

Today, the disease is widespread in farm and domestic animals in many parts of India. An outbreak of water-borne leptospirosis in and around Mumbai region occurred in July 2005, followed by record monsoon rainfall on 26th July 2005. Leptospirosis claimed most lives along with gastroenteritis, and nearly 2500 people were being treated in hospitals in the Mumbai area. At least 37 people died in Mumbai in 8 to 10 days and another 2 died in the adjoining district of Kalyan. The incessant monsoon rains lasting a week from June 26 were the worst in the known history of Maharashtra. During the floods, many people waded through water up to neck-deep to reach safety or get food supplies.

During this outbreak, samples were sent to Regional Medical Research Centre, Pune for diagnosis, identification and isolation of leptospires. Valuable diagnostic time was lost between the sending of sample and receiving a report. Therefore a Nodal Laboratory for Re-emerging Diseases was set up at the Department of Zoonosis, Haffkine Institute with the kind support from the Dhirubhai Ambani Foundation and Medical Education

Chain Reaction PCR (Real Time PCR) as well as IgM ELISA methods.

Although modern diagnostic tools including Polymerase Chain Reaction technique be able to provide quicker results, MAT is still considered the gold standard. This not only extremely labour intensive, but also requires vast expertise and maintenance of leptospiral cultures.

Another area that needs more light to be shed on is the prevalence of leptospirosis in animals. Data reflecting the source of infection, specifically in the Mumbai region, has been scattered. Hence, the present study was carried out to determine prevalence of leptospirosis in humans and animals using conventional and modern diagnostic tools. A comparison of various conventional and modern tools for the diagnosis of Leptospirosis was done with emphasis on the sensitivity and specificity compared to the gold standard. Prevalence studies on humans and animals were aimed at a comprehension of the risk groups/factors involved and to study the transmission dynamics via zoonotic routes. The study also focused on identifying the serovar prevalent in and around Mumbai, which would be of epidemiological importance.

*Review
Of
Literature*

Historical aspects

Leptospirosis is a Zoonosis of ubiquitous distribution, caused by infection with pathogenic *Leptospira* species. The spectrum of human disease caused by leptospirosis is extremely wide, ranging from subclinical infection to a severe syndrome of multi-organ infection with high mortality. This syndrome, icteric leptospirosis with renal failure, was first reported over 100 years ago by Adolf Weil in Heidelberg.[55] The disease was fully described in 1887 by Goldsmidt for a syndrome of severe febrile illness, jaundice and renal abnormalities.[56]

However, an apparently identical syndrome occurring in sewer workers was described several years earlier. Earlier descriptions of diseases that were probably leptospirosis were reviewed recently. Leptospirosis was certainly recognized as an occupational disease of rice harvesting in ancient China (*faine*), and the Japanese name *akiyami*, or a fever, persists in modern medicine. With hindsight, clear descriptions of leptospirosis with jaundice can be recognized as having appeared earlier in the 19th century, some before the description by Weil.[37]

The etiology of leptospirosis was demonstrated independently in 1915 in Japan and Germany (207). In Japan, Inada and Ido detected both spirochetes and specific anti-leptospiral antibodies in the blood of Japanese miners with infectious jaundice, and two groups of German physicians studied German soldiers afflicted by “French disease” in the trenches of northeast France. Uhlenhuth and Fromme and Hubener and Reiter detected spirochetes in the blood of guinea pigs inoculated with the blood of infected soldiers.[55] Confirmation of the occurrence of leptospirosis on both sides of the Western Front was obtained in 1916 after the publication in Europe of Inada’s work.[57] Given the initial controversies

the kidney tubules of a patient who reportedly died of yellow fever. [37] The spirochete had hooked ends, and Stimson named them *Spirochaeta interrogans* because of its resemblance to a question mark. The importance of occupation as a risk factor was recognized early. The role of the rat as a source of human infection was discovered in 1917 [35], while the potential for leptospiral disease in dogs was recognized, but the distinction between canine infection with *L. interrogans* serovars icterohaemorrhagiae and canicola took several years. Leptospirosis in livestock was recognized some time later.[58] Several monographs provide extensive information on the early development of knowledge on leptospirosis.[37, 59, 60]

2.1 Taxonomy and Classification

Taxonomic status-

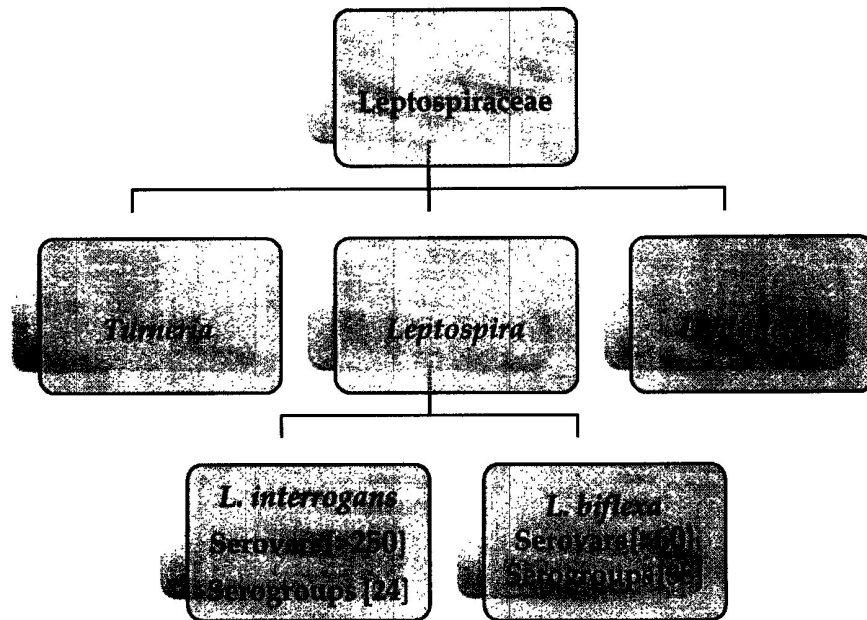
Order: spirochaetales.

Family: leptospiraceae.

Genus: *Leptospira*

Species

1. *L. interrogans*
2. *L. biflexa*



Prior to 1989, the genus *Leptospira* was divided into two species, *L. interrogans*, comprising all pathogenic strains, and *L. biflexa*, containing the saprophytic strains isolated from the environment. *L. biflexa* was differentiated from *L. interrogans* by growth of the former at 13°C and growth in the presence of 8-azaguanine (mg/mL).[62]

Prior to 1989, the genus *Leptospira* was divided into two species, *L. interrogans* comprising all pathogenic strains, and *L. biflexa*, containing the saprophytic isolated from the environment.

Both *L. interrogans* and *L. biflexa* are divided into numerous serovars defined by agglutination after cross-absorption with homologous antigen. If more than 10% homologous titre remains in at least one of the two antisera on repeated testing, strains are said to belong to different serovars. Over 60 serovars of *L. biflexa* have been recorded. Within the species *L. interrogans* over 200 serovars are recognized; additional serovars have been isolated but have yet to be validly published. Serovars that are antigenically related have traditionally been grouped into serogroups.[63] Serogroups have no taxonomic standing; they have proved useful for epidemiological understanding. The serogroups of *L. interrogans* and some common serovars are listed in Table 1.

Serogroup	Serovar(s)
Icterohaemorrhagiae	Icterohaemorrhagiae, copenhageni, lai, zimba
Hebdomadis	hebdomadis, jules, kremastos
Autumnalis	autumnalis, fortbragg, bim, weerasinghe
Pyrogenes	pyrogenes
Bataviae	bataviae
Grippotyphosa	grippotyphosa, canalzonae, ratnapura
Canicola	canicola
Australis	australis, bratislava, lora
Pomona	pomona
Javanica	javanica
Sejroe	sejroe, saxkoebing, hardjo
Panama	panama, mangus
Cynopteri	cynopteri
Djasiman	djasiman
Sarmin	Sarmin
Mini	mini, Georgia
Tarassovi	tarassovi
Ballum	ballum, aroborea
Celledoni	celledoni
Louisiana	louisiana, lanka
Ranarum	ranarum
Manhao	manhao
Shermani	shermani
Hurstbridge	hurstbridge

The phenotypic classification of leptospires has been replaced by a genotypic classification which includes a number of genomospecies including all serovars of both *L. interrogans* and *biflexa* (Table 2). Genetic heterogeneity was demonstrated and was demonstrated soon ago[64] and DNA hybridization studies led to the definition of several genomospecies of *Leptospira*. [65] After an extensive study of several hundred strains by CDC, leptospires are recently defined as 17 genomospecies. [58] Historically, the first methods used were DNA-DNA homology measured by DNA hybridization, which remains a reference method for typing bacterial species. DNA from different leptospires was hybridized and homology ratios under various conditions of stringency calculated. Leptospires were then grouped into several genomospecies, coinciding with groups according to G+C % ratios, each relatively homogeneous and clearly separate from others. [37] Since it is currently not practical to use DNA-DNA hybridization for routine identification at the species level, more recent, less difficult and faster methods that provide essentially the same information are used. Generally, they employ PCR-based strategies followed by endonuclease restrictions of amplified DNA target or by hybridizing leptospiral chromosomal DNA. Mapped restriction site polymorphism (MRSP) has been used with PCR amplification of 16S rRNA and 23S rRNA genes for classification and taxonomic purposes, [66] allowing reference strains to be grouped by their species in agreement with DNA relatedness studies.

The first method used widely was restriction endonuclease analysis of chromosomal DNA by fixed field electrophoresis, but it is limited because the number of restriction fragments is too high to be easily readable. Interestingly the patterns produced by

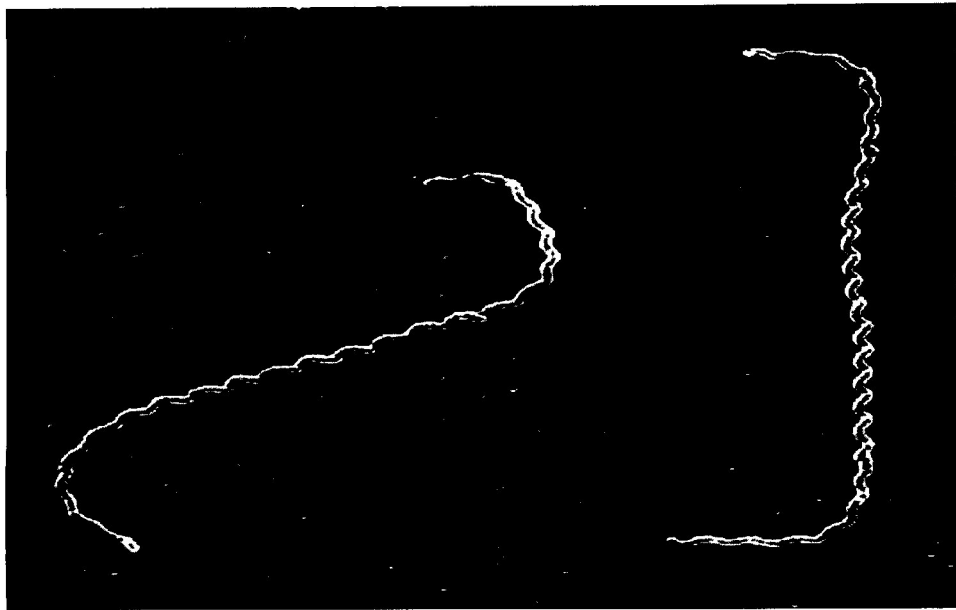
The data support that the molecular evidence has the validity of discriminating leptospiral strains almost identical to that provided through serovar concept. Random amplification of chromosomal DNA with its different variant has random amplification of polymorphic DNA analysis (RAPD) or arbitrarily primed PCR (AP-PCR) have yielded overall similar results. Specific probes, obtained from DNA fragments or by synthetic oligonucleotide, were also used to identify specific serovars and sub-serovar variants. Currently, a set of ribotypes is available for reference strains. Two probes derived from the serovar hardjo strain hardjopravitno, containing repetitive sequences designed as pL590 and pL591 were used to categorize groups consistent with the general classification based on DNA-DNA hybridization. Other probes or repetitive sequence elements for identification of strains have been described for hardjovovis and other cattle related leptospires.[5]

Both phenotypic classifications based on serology and genotypic classification of leptospires revealed problematic discrepancies. The term “*Leptospira interrogans* (sensu lato)” is used for the broad group of leptospires with animal host associations, contrasted with “*L. interrogans* (sensu stricto)”, used for the genomospecies. The term “biflexa” is also used to describe the general group of largely non-pathogenic, free living leptospires, sometime referred to as “*Leptospira biflexa* (sensu lato)”, in contrast to the genomospecies *Leptospira biflexa* (sensu stricto).[68]

<i>L. interrogans</i>	Icterohaemorrhagiae, Canicola, Pomona, Australis, Autumnalis, Pyrogenes, Grippotyphosa, Djasiman, Hebdomadis, S Bataviae, Ranarum, Louisiana, Mini, Sarmin
<i>L. noguchii</i>	Panama, Autumnalis, Pyrogenes, Louisiana, Bataviae, Tara Australis, Shermani, Djasiman, Pomona
<i>L. santarosai</i>	Shermani, Hebdomadis, Tarassovi, Pyrogenes, Autun Bataviae, Mini, Grippotyphosa, Sejroe, Pomona, Jav Sarmin, Cynopteri
<i>L. meyeri</i>	Ranarum, Semarang, Sejroe, Mini, Javanica
<i>L. wolbachii</i>	Codice
<i>L. biflexa</i>	Semarang, Andamana
<i>L. fainei</i>	Hurstbridge
<i>L. borgpetersenii</i>	Javanica, Ballum, Hebdomadis, Sejroe, Tarassovi, Celledoni, Pyrogenes, Bataviae, Australis, Autumnalis
<i>L. kirschneri</i>	Grippotyphosa, Autumnalis, Cynopteri, Hebdomadis, Aus Pomona, Djasiman, Canicola, Icterohaemorrhagiae, Batavia
<i>L. weilii</i>	Celledoni, Icterohaemorrhagiae, Sarmin, Javanica, Tarassovi, Hebdomadis, Pyrogenes, Manhao, Sejroe
<i>L. inadai</i>	Lyme, Shermani, Icterohaemorrhagiae, Tarassovi, M Canicola, Panama, Javanica
<i>L. parvac</i>	Turneria
<i>L. alexanderi</i>	Manhao, Hebdomadis, Javanica, Mini

Leptospires are tightly coiled spirochetes, usually 0.1×6.0 μm to 0.1×20 μm . occasional cultures may contain much longer cells. The helical amplitude is approximately 0.1 to 0.15 μm , and the wavelength is approximately 0.5 μm . [37] cells have pointed ends, either or both of which are usually bent into a distinctive shape shown in Figure 2.

Figure 2: Scanning electron micrograph of *L. interrogans* bound to a 0.2- μm membrane filter



(Adapted from Open Access Article: Molecular Epidemiology of Leptospirosis in the Amazon)[69]

Two axial filaments (periplasmic flagella) with polar insertions are located in the periplasmic space. The structure of the flagellar proteins is complex.[70] Leptospira exhibit two distinct forms of movement, translational and non-translational. Leptospira have a typical double membrane structure in common with other spirochetes, in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and overlain by an outer membrane.



carbol fuchsin counterstain.[73]

Leptospire are obligate aerobes with an optimum growth temperature of 28–30°C. They grow in simple media enriched with vitamins (vitamins B2 and B12 are growth factors), long-chain fatty acids, and ammonium salts. Long-chain fatty acids are utilized as the sole carbon source and are metabolized by β -oxidation[74].

The initiation of the growth of parasitic leptospire is enhanced by pyruvate, an essential nutrient. Because of this, leptospire are resistant to the pyrimidine analog fluorouracil, which inhibits growth of most other bacteria and hence it is used in isolation of the organism from contaminated cultures. Leptospire metabolize fatty acids by oxidation and the metabolic end products are acetate and dioxide.[68]

2.5 Culture Methods

Leptospire grows in media containing serum or albumin plus polysorbate and in protein-free synthetic media. Several liquid media containing rabbit serum have been described by Fletcher, Korthoff, Noguchi, and Stuart.[75] The most widely used medium in clinical practice is based on the oleic acid-albumin medium EMJH (Ellinghausen, McCullough, Johnson and Harris) which contains Tween 80 and bovine serum albumin. [76] Some strains are more fastidious and require the addition of either pyruvate [77] or rabbit serum for initial isolation. Addition of 5-fluorouracil is found to inhibit the growth of contaminants from clinical specimens.[78] Other antibiotics have been added to the media for culture of veterinary specimens, in which contamination is more likely to occur. Growth of leptospire is often slow on primary isolation, and cultures are retained for up to 13 weeks before being discarded, but pure subcultures in liquid media usually

medium, which becomes increasingly turbid as incubation proceeds. This growth is related to the optimum oxygen tension and is known as a Dinger's ring or Dinger's ring. Leptospiral cultures may be maintained by repeated subculture or preferably by storage in semisolid agar containing hemoglobin.[37] Long-term storage by lyophilization is also used. Growth on media solidified with agar has been reported. Colonial morphology is dependent on agar concentration and serovar. Media can also be solidified using gelatin.[80] Solid media have been used for isolation of leptospires, to separate cultures of leptospires, and for detection of hemolysin production.[55]

2.6 Molecular Biology

Leptospires are phylogenetically related to other spirochetes.[81] The leptospiral genome is approximately 5000 kb in size[82], although smaller estimates have been reported. The genome is comprised of two sections, a 4400-kb chromosome and a smaller 200-kb chromosome.[37] Leptospires contain two sets of 16S and 23S rRNA genes but only one 5S rRNA gene and the rRNA genes are widely spaced.[83] The study of leptospiral genetics has been slowed by the lack of a transformation system. Recently, a shuttle vector was developed using the temperate bacteriophage LE1 from *L. biflexa*. [84] This advance offers the prospect of more rapid progress to understand *Leptospira* at the molecular level. Several repetitive elements have been identified [85-87], of which several are insertion sequences (IS) coding for transposases. IS1533 has a single reading frame, while IS1500 has four. Both IS1500 and IS1533 are found in many serovars, but the copy number varies widely between different serovars and is

been cloned and analyzed, including several for amino acid synthesis [89], rRNA, ribosomal proteins [91], RNA polymerase [92], DNA repair, heat shock proteins, sphingomyelinase [93], hemolysins [94], outer membrane proteins [95], flagellin [96], and lipopolysaccharide (LPS) synthesis.[85] Within *S. icterohaemorrhagiae*, the genome appears to be conserved.[97] This conservation allowed the identification of at least one new serovar by recognition of distinct pulsed-field gel electrophoresis (PFGE) profiles.[98] However, the recent demonstration of heterogeneity within serovar indicates the need for further study of multiple isolates of individual serovars.[99]

2.7 Epidemiology

Leptospirosis is presumed to be the most widespread zoonosis in the world. The source of infection in humans is usually either direct or indirect contact with the urine of an infected animal. The incidence is significantly higher in warm climate countries than in temperate regions; this is due mainly to longer survival of leptospires in the environment in warm, humid conditions. However, most tropical countries are also developing countries, and there are greater opportunities for exposure of the human population to infected animals, whether livestock, domestic pets, or wild or feral animals. The disease is seasonal, with peak incidence occurring in summer or fall in temperate regions, where temperature is the limiting factor in survival of leptospires, and during rainy seasons in warm-climate regions, where rapid desiccation would otherwise prevent survival. In the developed world, the incidence has come down substantially and most cases that

Human infection results from exposure to infected urine of carrier or reservoir animal either directly or via contamination of soil or water. Animals, including humans, are divided into maintenance hosts and accidental (incidental) hosts. The disease is maintained in nature by chronic infection of the renal tubules of maintenance hosts. A maintenance host is defined as a species in which infection is endemic and is 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. Other animals (such as humans) may become infected by indirect contact with the maintenance host. Animals may be maintenance hosts of some serovars and accidental hosts of others, infection with which may cause severe or fatal disease. The most important maintenance hosts are small mammals, which may transfer infection to domestic farm animals, dogs, and humans. The extent to which infection is transferred depends on many factors, including climate, population density, and the degree of contact between maintenance and accidental hosts. Different rodent species may be reservoirs for distinct serovars, but rats are generally maintenance hosts for serovars of the serogroup Icterohaemorrhagiae and Ballum, and mice are the maintenance hosts for serovars of Ballum. Domestic animals are also maintenance hosts; dairy cattle may harbor serovars hardjo, pomona, and grippityphosa; pigs may harbor pomona, tarassovi, or bratton; sheep may harbor hardjo and pomona; and dogs may harbor canicola.[100] Differences in variations in maintenance hosts and the serovars they carry occur throughout the world. Information about the prevalent serovars and their maintenance hosts is essential to understand the epidemiology of the disease in any region.

exposures. Occupation is a significant risk factor for humans. Direct contact with ir animals accounts for most infections in farmers, veterinarians, abattoir workers inspectors, rodent control workers and other occupations which require contac animals. Indirect contact is important for sewer workers, miners, soldiers, septi cleaners, fish farmers, gamekeepers, canal workers, rice field workers, taro fa banana farmers and sugar cane cutters. Miners were the first occupational risk gr be recognized.[55] The occurrence of Weil's disease in sewer workers was first re in the 1930s.[101] Serovar icterohaemorrhagiae was isolated by guinea pig inoc from patients, from rats trapped in sewers and from the slime lining the sewer Glasgow, Scotland, a seroprevalence among sewer workers of 17% was reported Fish workers were another occupational group whose risk of contracting leptos was recognized early. Between 1934 and 1948, 86% of all cases in the northe Scotland occurred in fish workers in Aberdeen.[102] Livestock farming is a occupational risk factor throughout the world. The highest risk is associated with farming and is associated with serovar hardjo [103, 104], in particular with milk dairy cattle. Significant risk of acquiring leptospirosis is also associated with recre exposures occurring in water sports, including swimming, canoeing , white water r [105] fresh water fishing, and other sports where exposure is common, such as pot and caving.[106] The potential for exposure of large numbers of individuals during competitive events. Several outbreaks of leptospirosis associated with wate been reported.[13]

Leptospirosis is a worldwide zoonosis. According to the occupational groups involved and the nature of the disease presentations, different names have been used, e.g. day fever found commonly in Japan, Cane cutter's disease in Australia, Rice Leptospirosis in Indonesia and Fort Bragg fever, which appeared as an outbreak in the US. Weil's disease, which is one of the severe forms of this disease, occurs in many countries, including India and other South-East Asian Countries, China, continental Europe and England. Leptospirosis exists in all the five inhabited continents and a large number of countries. It occurs in tropical, subtropical and temperate zones.

In November 1961, an outbreak of Leptospirosis occurred among 186 US Army soldiers in the canal-zone who had engaged in a jungle exercise 10–13 days earlier. Epidemiological investigations (1975–1977) carried out in Barbados revealed the seroprevalence of Leptospirosis in the various occupational groups to be 29.8% (100% in sanitation workers – 42.7% followed by sugarcane workers – 39.4%). Fever cases suspected leptospirosis gave seropositivity of 28.7% in a hospital survey and seropositivity was noted in healthy individuals.[107] In a survey made in northern Trinidad between mid 1977 and mid 1978 leptospiral infection was found to be widespread in the general population, and among occupational groups the highest prevalence of antibodies was found in sugarcane workers (45% infected). From 1979 to 1982, sera were collected from fever cases in Trinidad; 9% were confirmed as leptospirosis cases and 23% showed evidence of previous infection. A high prevalence of leptospiral antibodies in humans was reported from Somalia in 1982.[108]

Another survey in 1987 in Italy showed prevalence in rural areas of 11.34%, while

Trinidad school children aged 7 to 14 years.[109] The Hawaii State Department of Health reported a leptospirosis incidence rate of 2.97 per 100000 populations, compared with the national rate of leptospirosis in the United States of 0.02 per 100000 in 1992. The proportion of leptospirosis cases in Hawaii related to occupational exposure decreased from 56% during 1971–1975 to 29% during 1986–1990, whereas cases related to recreational, habitation or vocational exposure increased from 43% during 1971–1975 to 71% during 1986–1990. Three papers have been published in Uruguay on Acute Renal Failure (ARF) in Leptospirosis. The first series of five cases was published in 1992. These five cases represented 8% of the total cases.[108]

The second series of 20 cases, published in 1993, showed an incidence of ARF of 0.7–1.3 cases per 100000 per year. The expected frequency of ARF in Uruguay is 0.7–1.3 cases per 100000 per year. Symptomatic leptospirosis is particularly frequent and severe in the Seychelles; 80 cases were reported over a two-year period during 1989–1990, 65 cases during 1993–1994 and 75 cases during 1995–1996.[110]

In October, 1995, epidemic hemorrhagic fever, without jaundice or renal manifestations, was reported to be caused by leptospira in rural Nicaragua, following flooding.[111]

In 1995, 90 out of 295, i.e. 30.5%, of apparently healthy individuals tested positive for anti-leptospira antibodies by MAT in the Cordillera province of Bolivia.[112]

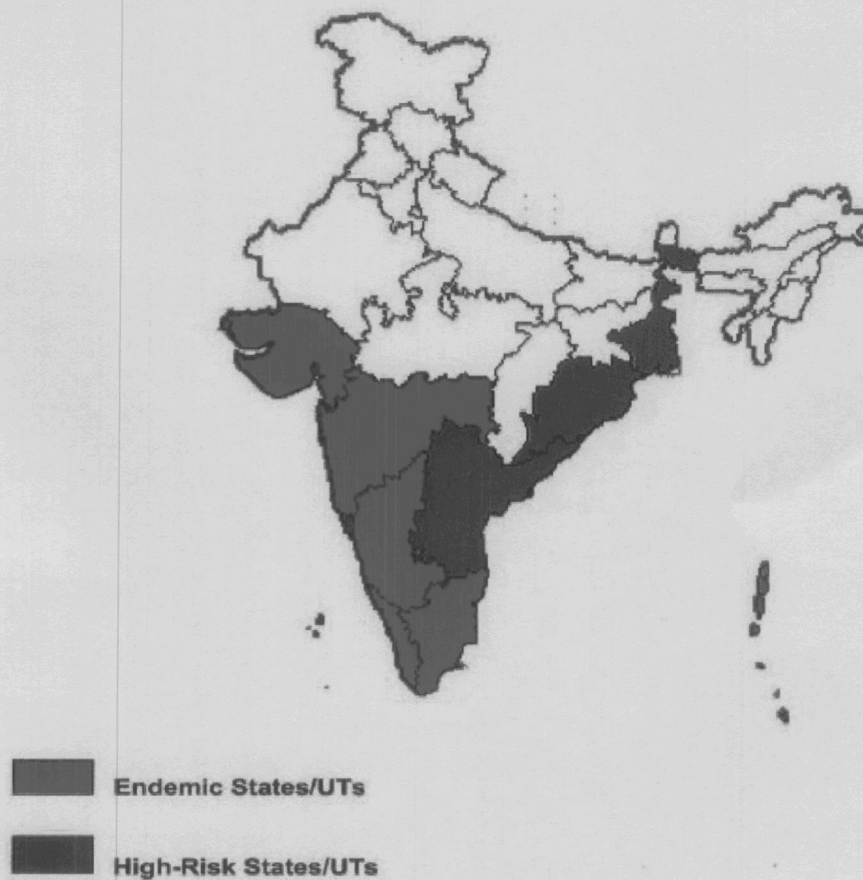
In Turkey, screening of 1440 people for leptospira antibodies using MAT revealed 13.9% positivity. Among rice field workers seropositivity was found to be 9.6–13.9%. In Vientiane, Laos serological evidence of recent leptospiral infection in 1995 and 1996 was found in 13.9% of 1440 people screened.

An outbreak of leptospirosis among white-water rafters in Costa Rica was reported in September, 1996.[114] An outbreak of acute febrile illness in 1998 among athletes participating in triathlons in Wisconsin and Illinois was reported to be caused by leptospirosis.[115]

2.9 The Indian Scenario

The Leptospirosis situation in India is a cause of concern. The endemicity of the disease is expanding to cover more and more states in the country. Geographic distribution of Leptospirosis in the country is depicted in Figure 3.

Map of India showing Leptospira Endemic and High-Risk States/UTs



(Adapted from Open Access Article: Report of the Brainstorming Meeting on Leptospirosis Prevention and Control)

The disease is presently endemic and deeply entrenched in Gujarat, Maharashtra, Karnataka, Kerala, Tamil Nadu and Andaman & Nicobar Islands. High risk areas include Goa, Andhra Pradesh, Orissa and West Bengal. Table 3 clearly shows, the outbreak reported in different states/ Union Territories (UTs) from 1984–2005.

Year	State
1984	Tamil Nadu
1988	Andaman & Nicobar (A & N)
1994	Gujarat
1995	Gujarat
1997	Gujarat, A & N
1999	Gujarat, Tamil Nadu
2000	Maharashtra, Gujarat, Tamil Nadu, Kerala
2001	Maharashtra, Gujarat, Tamil Nadu, Kerala & Goa
2002	Kerala, Maharashtra, Gujarat, Tamil Nadu
2003	Kerala, Gujarat, Tamil Nadu, A & N
2004	Kerala, Gujarat, A & N
2005	Maharashtra, Kerala, Gujarat, Tamil Nadu, A & N

The disease is presenting a cycle of epidemics to increasing areas of endemicity resulting in further outbreaks. Epidemics are associated with changes in human behavior, and sewage contamination of water and changes in animal reservoir density. A cause for concern is the increasing outbreaks of leptospirosis in post-disaster situations such as the Orissa super-cyclone and floods in 1999 and Mumbai flash floods in 2005.

The serological study of leptospirosis in man has been limited in India. For the first time in India, Taylor and Goyale reported the isolation of leptospira organisms from patients showing Jaundice in Andaman Islands during 1931[6]. Soon after this report, they reported isolation of leptospira interrogans serovar icterohaemorrhagiae from domestic animals in Chennai city. Since then several reports have emerged confirming prevalence of leptospirosis in various animal species and man in India.[117] In 1960, serological studies were conducted in various parts of India, showing the prevalence of leptospirosis in various animal species and man in India.

In 1966, out of 93 sera from Pyrexia of Unknown Origin (PUO) cases, 3 were positive for the agglutination lysis test, 1 against icterohaemorrhagiae and 2 for canicola, and 43 cases of jaundice, 2 were positive for icterohaemorrhagiae and 1 for icterohaemorrhagiae and pomona.[119]

In 1967, in Mumbai, 1 of 150 sera from infective hepatitis cases showed evidence of leptospira infection due to pyrogenes. Leptospira agglutinins at significant titres were demonstrated in 5 out of 17 sera from suspected cases of leptospirosis and in 6 cases of 11 sera from workers of animal farms and piggeries.[120]

In 1983, in Chennai, the seroprevalence of leptospirosis in jaundiced patients was 24% and it was 24% in PUO cases.[121] In 1983, a serological study was made in a population that consisted mainly of children in a village near Chennai, following an outbreak of disease in cattle; 35 of 75 (47%) human sera gave positive agglutination titres.[122]

During 1984–1985, acute renal failure due to leptospirosis in 19 human patients was reported in Chennai.[123]

In 1988, during the peak of the monsoon season, serum and urine samples from 54 patients, with a history of fever, vomiting, jaundice, abdominal pain and renal failure, from various hospitals in Chennai city and MAT revealed that 33 (82.5%) had specific leptospiral antibodies, with titres ranging from 1:160 to 1:6400 against different serotypes. Leptospirosis MAT titres 1:1600 and 1:800 occurred in 39 of 54 and 51 of 54 patients respectively, in patients admitted to the Government General Hospital, Chennai, during November and December 1990 to 1991 with symptomatology suggestive of disease.

manifestations and pulmonary involvement occurred in Diglipur of North Andaman during October to November 1993; 66.7% of the victims had significant titers of antibodies against leptospira.[124]

In 1994, an increase in the number of individuals with uveitis was noted at Aravind hospital, Madurai after an epidemic of leptospirosis in South India; the epidemic followed severe flooding of the Tamil Nadu District in the autumn of 1993; 46 patients (80%) had leptospiral DNA and 33/46 patients (72%) had positive serology. In 1995, a seroprevalence rate of 12% leptospirosis was found among febrile jaundice patients in Pondicherry.[125] Thirty-eight acute renal failure cases with a suspicion of leptospirosis were screened from July–November, 1996 and 27 seropositive cases were diagnosed by MAT.[126]

2.10 Geographic distribution in Mumbai

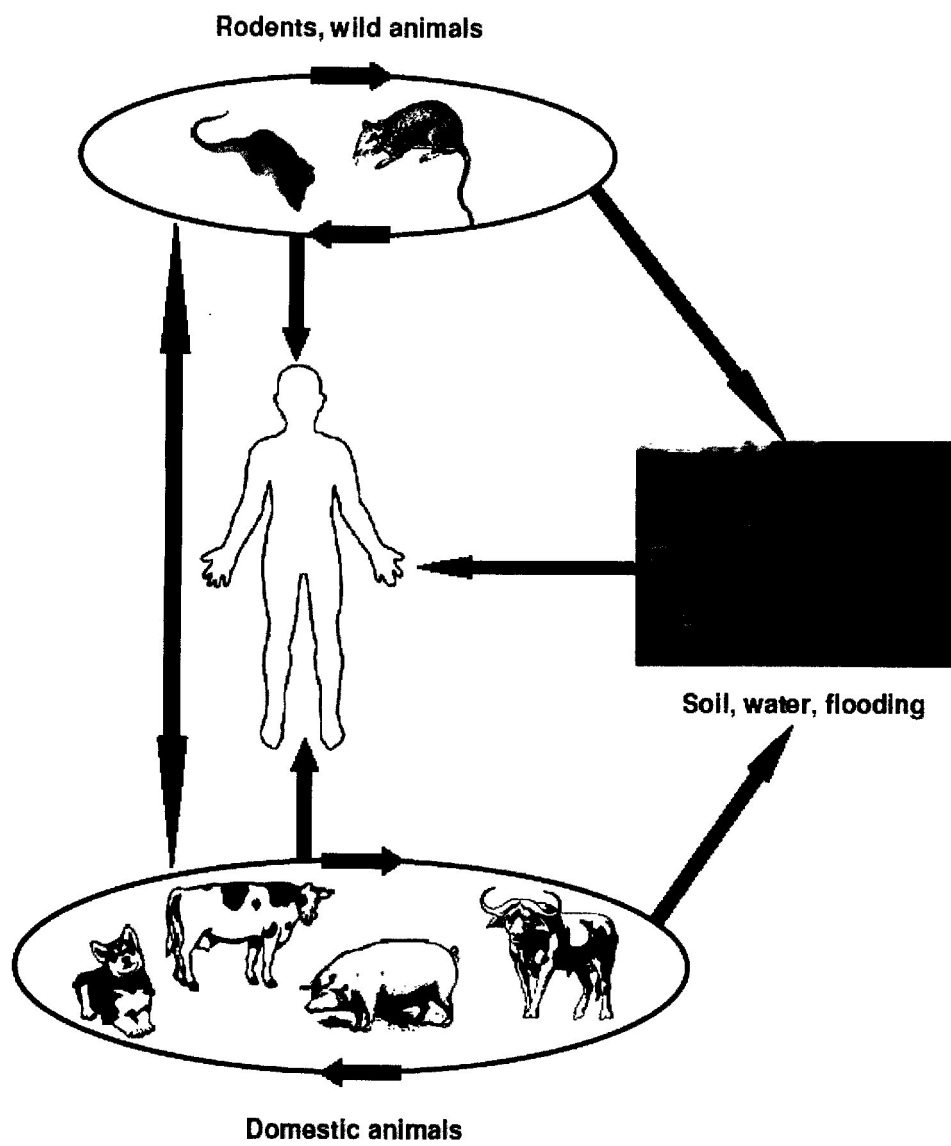
Mumbai lies on the west coast of India and has a deep natural harbour. It is the capital of the Indian state of Maharashtra. Mumbai is located at 18°58'30"N 72°49'30"E / 18.9750°N 72.8258°E / 18.9750; 72.8258 in the Indian state of Maharashtra. Mumbai consists of two distinct regions: Mumbai City and Mumbai Suburban District, which form two separate districts of Maharashtra. Mumbai, as an urban entity, spans a total area of 603.4 km² (233 sq mi), while Greater Mumbai, spans a total area of 437.7 km² (169 sq mi).

Mumbai has a tropical climate, specifically a tropical wet and dry climate, with months of dryness and peak of rains in July. Between June and September, the southwest monsoon rains lash the city. The maximum annual rainfall ever recorded was 3,412 mm (134 in) in 1974.

City, and 2,457 mm (96.73 in) in the suburbs. Leptospirosis is endemic in Mumbai. Climatic and environmental factors were probably responsible for epidemics of leptospirosis seen in Mumbai in the recent years.[127]

Lahiri et al. was the first person to identify and isolate leptospires from a human case. This was followed by Dalal et al. who reported 5 cases of leptospirosis in 1960.[118] After a relatively large interval, the disease emerged again in the city as a major problem in 1998–99, when there were 111 reported cases and 11 deaths. In the year 2000, the number of deaths raised up to 36 in Mumbai and surrounding district, which created panic. An outbreak occurred in suburban Mumbai in the year 2000. During this outbreak, serovar copenhageni was found to be responsible for most of the cases. Sunil Karande et al. detected acute leptospirosis in 30 (32%) children out of 93 admitted and suspected of leptospirosis confirming that an outbreak of leptospirosis occurred in Mumbai following heavy rainfall and flooding in 2001.[128] Maskey et al. have reported 31.8% seropositivity by rapid tests during the monsoon deluge in 2005.[129] During 2005 outbreak, Mathur et al., reported positivity of 29.9% by PCR. Commonest serovar found during this study was icterohaemorrhagiae followed by bataviae, tarassovi and pomona.[130] Regular prevalence is lacking in this geographical area and prevalent serovar has not been determined; detailed studies on prevalent serovars and sources of infection would definitely be of use since the disease is endemic in this area.

humans being accidental victims. The natural habitat of leptospires is the renal tubules of carrier animals. The animal hosts of leptospires are broadly categorized as carrier animals in whom the carrier state is temporary ranging from a few months to years, and reservoir hosts in whom the carrier state is life long. The reservoir hosts are the primary source of leptospires. They infect both animals and human beings. The carrier hosts who get infection either from reservoir hosts or from other carrier hosts can also act as the source of infection to human beings. Human to human transmission is very rare and leptospirosis infection in human beings is the dead end of transmission chain. Therefore, the source of infection both in animals and humans is the carrier animal, though infection may also occur through various environmental vehicles. Although leptospires are susceptible to various environmental factors, particularly drying, acidic pH, salinity and presence of detergents and other bactericidal agents, they can survive for long periods in water and wet soil under favourable conditions. Some serovars of leptospires have been found to survive retaining their infective potential in soil for up to 74 days.[131] There is indirect evidence that leptospires can grow and multiply in environment under favourable conditions. Thus environment plays an important role in the epidemiology of leptospirosis (Figure 4). Three basic factors i.e. the animal carriers, environment suitable for the survival of leptospires and exposure of people to contaminated environment are essential for the survival of leptospires and exposure of people to contaminated environment. Carrier animals, their urine, body fluids or tissue, are always involved in the transmission of leptospiral infection. However, the exact nature of these factors varies from one ecological setting to the other, thus making the transmission cycle complex and dynamic. Successful transmission depends on the presence of all these three factors.



(Adapted from Open Access Article: Leptospirosis in the Asia Pacific region [4])

2.12.1 Entry

Leptospire are presumed to enter via small abrasions, sodden skin or breaches surface integument. They may also enter directly into the bloodstream or lymphatic system through the conjunctiva; the genital tract in some animals; the nasopharyngeal mucosa, possibly through the cribriform plate; the lungs following inhalation of aerosols (rare); or through invasion of the placenta from mother to fetus at any stage of pregnancy in mammals. It is unlikely that penetration of intact skin or other mucosal surfaces occurs. Abraded or sodden skin may allow penetration of leptospire through its breach of integrity. There is no direct evidence that motility or chemotaxis enhance entry to skin although chemotaxis to red blood cells have been demonstrated.[132]

2.12.2 Survival

The ability to survive and grow in tissues is a major component of virulence.[132] Once leptospire enter through the integument, they are immediately exposed to the effects of non-specific factors of pH, redox potential, electrolytes, fatty acids and small organic molecules, some of which may be nutrients. Unlike pyogenic bacteria, leptospire cause an acute inflammatory response to their presence in tissues. Individual leptospire or significant clumps, can exist and multiply in natural or experimental infections without inflammation. They clearly do not activate recognition factors by interaction with mediators of inflammation. In some animals, auto-immunity accounts for the lesion of cellular infiltration in the kidney.[37]

Survival of leptospire in tissues in a naive animal is mediated by their resistance to innate immunoglobulins, in tissue fluids or plasma. These “natural antibodies” may

genetic makeup of the host species.[132] They act together with complement and lysozyme to lyse the leptospires following attachment to a postulated receptor on the leptospiral surfaces.

2.12.3 Spread and growth

Leptospires spread almost immediately from the site of entry, via lymphatics to the bloodstream, where they circulate to all tissues. Avirulent and non-pathogenic leptospires which reach the bloodstream are cleared rapidly, within minutes of entry, by reticulo-endothelial phagocytosis; the leptospires are found at first mainly in the lungs, later in the liver and spleen; those leptospires which survive by evading phagocytosis, by definition the virulent organisms in the inoculum, grow exponentially, with a doubling time of approximately 8 hours, in the bloodstream and tissues.[37]

2.12.4 Pathogenic mechanisms

The central lesion, characteristic of all forms of leptospirosis, is damage to the walls of small blood vessels, leading to leakage and extravasation of cells, including hemorrhages. The important properties or the virulence factors of the leptospires include Adhesion, Lipopolysaccharide and others:

Adhesion:

In 1984, Vinh et al. suggested that leptospires attach to the fibroblast cells in mouse fibroblast (1929) cells lines. They observed that within 3 hours of infection of cell

concluded that leptospires probably enter these cells through damaged membranes or by a phagocytosis-like-mechanism.[133] In experiments by Ballard et al. using primary cultures of mouse renal tubular cells, virulent strains of *L.interrogans* serovar copenhageni and ballam attached within 3 hours, while avirulent leptospires did not bind even after 24 hours.[134]

Yuri in 1993 showed that virulent strains exhibit chemotaxis towards haemoglobin and put forward the hypothesis that virulent leptospires can recognize haemoglobin in surface water, which is the most common habitat of leptospires, reach cuts and abrasions on the skin (perhaps ascending a gradient of haemoglobin), and invade the bodies of humans and animals.[135]

A fibronectin-binding protein specifically expressed on the surface of virulent *L. interrogans* serovar icterohaemorrhagiae, but not on avirulent strains, may be significant in initial adhesion and invasion at cutaneous or mucosal sites of entry.[96]

Consistent with the predicted ability to migrate through host tissues, *Leptospira* have a range of potential virulence factors that may facilitate this process.

Lipopolysaccharide (LPS):

Leptospiral Lipopolysaccharide(LPS) has structural, chemical and immunological properties resembling those of Gram-negative bacterial LPS. But leptospiral LPS is considerably less toxic than is typical Gram-negative LPS.

Lipopolysaccharide and the outer membrane protein OmpL1 are also implicated in interstitial nephritis. [136]

humans and animals, accompanied by independent fibrinolysis consistent disseminated intravascular coagulopathy. [137]

Others:

The haemolysins of serovars ballum, hardjo, pomona and tarassov are sphingomyelinases.

Haemolytic, sphingomyelinase, and phospholipase activities have been described *in vitro* and, subsequently, specific genes have been characterized including haemolysin, sphingomyelinase C, sphingomyelinase H, and haemolysis-associated protein-1 (also known as LipL32).[138] By contrast with sphingomyelinase C, sphingomyelinase H showed no sphingomyelinase activity but was shown to be a cytotoxic pore-forming protein on several mammalian cells.[139]

Strains of serovar pomona and copenhageni elaborate a protein cytotoxin, which *in vitro* elicited a typical histopathological effect with infiltration of macrophages and polymorphonuclear leukocytes. A cytotoxic activity has been detected in the plasma of the animals.[140]

Burth et al. in 1997 purified Na⁺K⁺ ATPase inhibitor in endotoxin of *L.interrogans* and showed that the inhibitory activity was associated with unsaturated fatty acids, particularly palmitic and oleic acids. However similar activity was demonstrated by another strain of *L. biflexa*. [141]

Several additional haemolysins and sphingomyelinase-like proteins and pore forming proteins have been identified in the genome sequence of serovar lai. The *in vitro* relevance of these potential virulence factors in the pathogenesis of leptospirosis has not been determined.

Since *Leptospira* regulate expression of proteins in response to environmental signals, particularly with differences in protein expression between *in vitro* and *in vivo* conditions, a fundamental question for efficient diagnostic and vaccine development would be to address which genes are expressed during infection compared with *in vitro* conditions. The *leptospira* immunoglobulin-like protein (Lip a) contains domains homologous to proteins with attachment and invasion functions, and is expressed *in vivo* but not *in vitro*. [68]

2.12.5 Persistence, privilege sites and carrier state

In animals which do not die from acute phase, leptospires may persist in certain tissues and following clearance from the bloodstream. These tissues include proximal renal tubules, brain, anterior chamber of the eyes and genital tract in swine, sheep and probably other animals. Here, leptospires protected from antibody in the circulation, can survive and grow. In the kidney exponential growth continues with maximum duration about 28 days after infection. In the renal tubule, leptospires migrate through the interstitial spaces and between the renal epithelial cells to settle on their brush border, attach to microvilli on the luminal surface of the tubules. Leptospires are excreted in the urine where they are concentrated by coating with antibody which originates from the immune system. Leptospires in carrier urine are coated with polysaccharide or protein reported to be of host origin, rendering them agglutinate by serum agglutinating antibody. [68]

Phagocytosis has been studied by measuring clearance of leptospires from blood by the reticuloendothelial system [142] and by direct observation, using silver stain, immunofluorescence and electron microscopy. Less direct but valid observations of phagocytosis and opsonisation have been made using chemiluminescence response of leukocytes.⁵⁹ There is no direct evidence for the chemotaxis of leukocytes to leptospires under usual conditions of the infection or experimentation.

Any type of phagocytes can ingest opsonised leptospires. Non-pathogenic or avirulent organisms are opsonised by natural antibody whereas virulent leptospires require addition of specific immunoglobulins, of any immunoglobulin class, reacting with surface LPS determinants. Specificity is strongly serovar-associated; cross-reaction between closely related serovars occurs, but almost only within the serogroups. There is no evidence that any other antigens are opsonic.[143] Clearance from the bloodstream by the reticuloendothelial system follows opsonisation, whether passively in laboratory studies or experimentally or following infection.[142] Ingested leptospires can be observed in phagocytic vacuoles in Kupffer cells in the liver and elsewhere. Phagocytes readily ingest leptospires after mixing together in laboratory studies. Killed non-pathogenic or avirulent leptospires are taken up readily. The opsonic immunoglobulins required for phagocytosis of virulent leptospires may also kill and lyse them.[143] Opsonin-mediated immunity transferred passively by immunoglobulins, are not animal species specific. Immunity to re-infection is a function of development, or availability of opsonic immunoglobulins reacting with specific oligosaccharide epitopes of LPS.

There is evidence that they are digested and do not survive long or persist in the host. However, virulent leptospires were able to enter cultured Vero cells and macrophages.

macrophages. Once inside a phagocyte vacuole, leptospire can be seen to lose its shape and assume the spherical form resembling a spheroplast, believed to non-viable at that site. [144]

2.13 Immune response:

Leptospirosis is often characterized by biphasic clinical course. The manifestations of the septicemic phase are due to leptospiremia. Improvements in clinical symptoms are closely associated with appearance of serologically detectable antibodies. However, studies have shown that after an asymptomatic period of 1–3 days, the onset of the second phase is marked by recurrence of fever, meningitis and neurological involvement. It is well known that in leptospirosis the signs and symptoms of renal and hepatic involvement appear in the immune phase when specific agglutinins begin to be detectable. As protective immunity to leptospirosis is serovar specific, it was formerly believed to be almost exclusively humoral. Antibody is important in determining resistance to experimental leptospiral infections in animals as stated by Adler in 1977.[145] Tong reported twofold to fourfold increase in IgM and rise in the levels of IgA and IgG in the sera of patients infected with leptospirosis. Myer's et al. in 1978 and Palit et al. in 1979 described the broadly reactive genus specific antigens.[146, 147] Data provided by Wang et al. in 1984 suggest that the protection afforded by specific antibody appears to be mediated through opsonizing properties, which enhance the ingestion and destruction of leptospire by phagocytic cells, or through a direct killing effect on leptospire in the presence of complement.[148]

experimental finding also supports the possibility that the presence of specific antibodies may worsen cellular lesion and that sub-agglutinating amounts of homologous antiserum enhances the attachment of virulent leptospires to cells [133]. Abdulkadar et al. in 2002 stated that the leptospiral severity might be associated with the intensity of humoral immune response. He concluded that not only the virulence of the infecting serovar but also intensity and rapidity of development of immune response contribute to the severity of the disease.[149]

Thrombocytopenia in the disease may be attributed to immune mechanisms as suggested by Davenport in 1989.[150] Rugman in 1991 reported that increased titres of anti-cardiolipin antibodies are present in leptospirosis patient with severe complications *but not* in those with uncomplicated leptospirosis.[151]

However, considering induced immune response, despite serovar specificity, protective immunity to leptospirosis is not exclusively humoral. Merien et al. in 1992 reported that virulent leptospires induce apoptosis in vivo and vitro.[144]

Naiman et al. evaluated the cellular immune response induced by a protective monovalent serovar Hardjo vaccine in cattle. From their studies a potent Th1-type immune response and the involvement of $\gamma\delta$ T cells were clearly evident.[152] Recent data also establishes that pathogenic leptospires can stimulate in vivo the production of type 1 cytokines involved in cellular immunity by different methods.[153, 154]

Humans are incidental hosts of leptospira and are at high risk of contracting the disease when they come into contact with infected animals or their products. Some examples of risky activities include managing domestic and farm animals; milking cattle; assisting with births, removing stillbirths or removal of abortion products; dressing carcasses; cleaning urine spill from dogs and practicing as veterinarians and meat inspectors. Many cases are documented of infection in livestock farmers, veterinarians, abattoir workers, disease control workers and zoologists or other infection which involve direct or indirect contact with animals.

Indirect infections are also common in workers who are exposed to environments contaminated with urine from excretor animals. In Asian countries the practice of rice planting can lead to infection of rice planters via abraded skin, as their arms and legs are constantly being immersed in water contaminated by urine from rodents, buffaloes and pigs. Other occupational risk groups are sewer workers, miners, septic tank cleaners, canal workers, banana farmers and workers and sugar cane cutters.

There is also a significant risk associated with certain recreational activities associated with water sports, including swimming, canoeing, white water rafting and kayaking. Many of these outbreaks have followed extended periods of hot, dry weather, when pathogenic leptospires have presumably multiplied in freshwater ponds or rivers.

Sporadic cases of leptospirosis in tropical regions are acquired following exposure to contaminated water. They occur during daily activities, and are particularly associated with high seasonal rainfall. For example, infections have resulted from walking barefoot in damp conditions, from gardening with bare hands, from swimming or bathing in contaminated water and from accidental ingestion of contaminated water. Inhalation of contaminated aerosols of

significant reservoir for human infection in many tropical countries and may be an important source of outbreaks.

2.14.1 Clinical leptospirosis in humans:

The clinical manifestations of leptospirosis vary greatly. The incubation period is usually 7–14 days but may range from 2–21 days.

Most of the complications of leptospirosis are associated with localization of leptospire within the tissues during the immune phase and thus occur during the second week of the illness.[55] Sub-clinical and mild infections are quite common. Only a small proportion of cases develop severe leptospirosis.

2.14.2 Anicteric febrile illness

The great majority of infections caused by leptospire are either sub-clinical or of very mild severity, and patients will probably not seek medical attention. A smaller proportion of infections, but the overwhelming majority of the recognized cases, present with a febrile illness of sudden onset. The typical leptospirosis is biphasic illness.[55] The first phase which is called as septicemic or leptospiremic phase is characterized by acute systemic infection and by the presence of leptospira in the blood and cerebrospinal fluid. This phase lasts for 4–7 days followed by a 1–3 days of afebrile and asymptomatic period and then the second or immune phase starts with occurrence of fever and presence of leptospira in the urine. The onset is abrupt with rapidly rising fever, which is associated with chills and rigors, increasing prostration, severe headache and body ache.

frontal, less often retro-orbital but occasionally may be bi-temporal or occipital.

Body pains are most marked in the lower limbs especially in the calves and

Severe pain in the back, neck, abdomen and upper limbs is frequent. Prostration is

so marked that the patient has to stop work and frequently takes to bed. Anorexia, is

and vomiting are frequent and may be associated with constipation or diarrhoea.

Epistaxis may occur during the early stage. Chest pain, dry cough and haemoptysis

occur. Mental symptoms of restlessness, confusion, delirium and hallucinations

occasionally psychotic behaviour may be prominent features in some patients.

patients develop acute gastroenteritis with abdominal pain, vomiting and diarrhoea.

most characteristic findings on examination are conjunctival suffusion and severe myalgia.

Conjunctival suffusion occurs in the first three days and lasts from one day to more

week. It is bilateral; most marked on the palpebral portion and is usually associated

unilateral or bilateral sub-conjunctival haemorrhage. There is no inflammatory exudate

and true conjunctivitis does not occur. Myalgia is severe, even touching the limbs

causes intense pain. It is most commonly observed in the lower limbs especially

calves. A transient macular, maculopopular, erythematous, purpuric or urticarial rash

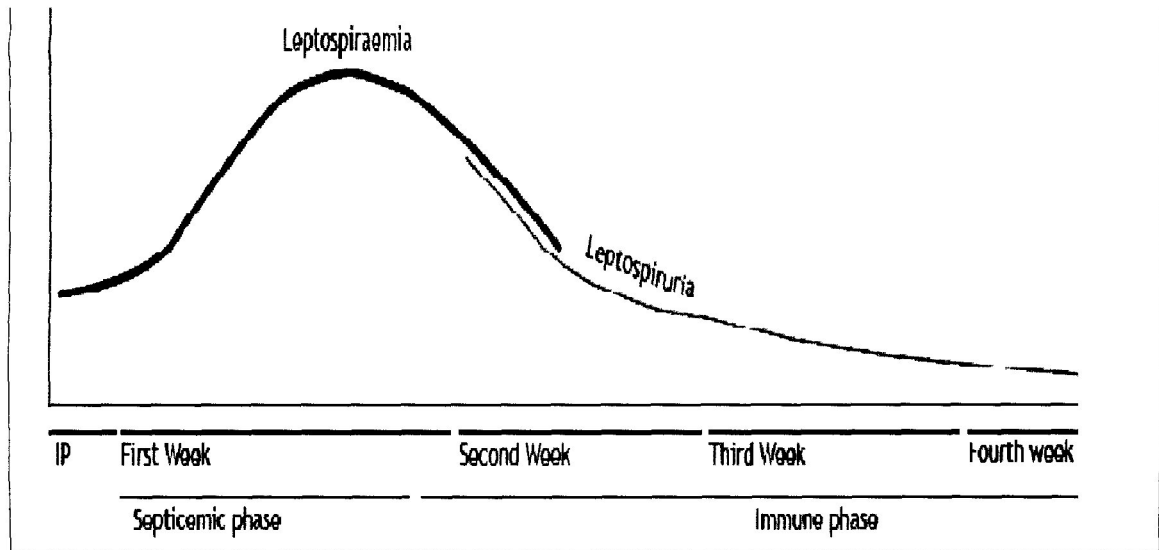
occur, usually on the trunk but it may be localized on the upper limbs or the shins.

septicemic phase subsides after 4–7 days. The temperature then becomes normal and

patient feels well. The second or immune phase is characterized by severe headache

to meningeal involvement and low grade fever. This lasts for 4–30 days or longer.

This biphasic course may not be seen in all patients as shown in Figure 5.



(Adapted from Open Access Article: Leptospirosis-Laboratory manual [61])

2.14.3 Icteric leptospirosis (Weil's Disease)

The severe form called Weil's syndrome comprises of icterus, renal and vascular involvement. It is rapidly progressive and severe. In illness with renal failure, jaundice is the most important clinical feature of the severity of illness. Jaundice occurs between the fourth and sixth day but may occur as early as the first week in some patients. The septicemic phase instead of subsiding progresses to a severe icteric second day or as late as the ninth day and deepens rapidly reaching a peak within a week. The liver is often enlarged and tender. Jaundice is due to hepatocellular necrosis, intra-hepatic cholestasis and increased bilirubin load from absorption of tissue haemorrhage. Marked elevations of bilirubin and mildly elevated transaminases are characteristics. Death rarely occurs due to liver failure. Renal involvement is the most serious complication and is the most common cause of death in icteric leptospirosis. Oliguria occurs in the second week but may occur as early as the fourth day of illness. A significant number of patients develop non-oliguric renal failure and have a better prognosis than oliguric renal failure. Renal manifesta-

to severe renal failure. Anorexia and vomiting worsen and hiccups may occur. Meningeal symptoms are frequent but overshadowed by hepatic and renal features. Confusion, restlessness, hallucinations, delusion and convulsions may occur. Severe bleeding, cardiac and pulmonary complications are frequent. Towards the end of the second week the patient is deeply jaundiced, uraemic, and haemorrhagic and become comatose. Death may occur at this stage or early in the third week from renal failure. Occasionally sudden death may occur from arrhythmias, cardiac failure or adrenal haemorrhage. Massive bleeding from the alimentary and respiratory tract may also end fatally. Death is rare due to hepatic failure but is virtually limited to icteric cases and in severe cases mortality may be as high as 15–40%. In those who are not severely ill, recovery takes place in the second week. Diuresis occurs and the blood urea level falls gradually. Fever subsides and the general conditions improve, however, jaundice takes a longer time to clear.[155]

	First stage	Second stage	First stage	Second stage
	3–7 days	0–30 days	3–7 days	0–30 days
	(Septicemic)	(Immune)	(Septicemic)	(Immune)
Clinical Findings	Fever, myalgia, headache, conjunctival suffusion, abdominal pain, vomiting	Meningitis, uveitis, rash, fever	High fever, Jaundice, Hemorrhage, Renal failure	
Leptospire Present in	Blood Cerebrospinal Fluid (CSF)	Urine	Blood CSF	Urine

2.14.4 Haemorrhagic pneumonitis

Pulmonary haemorrhages usually occur in the second week of severe forms of icteric leptospirosis. Occasionally it may occur within 24–48 hours of the onset of illness. The onset is sudden with rapidly raising fever which is associated with headache, generalized bodyache and cough which is dry in the beginning but becomes streaked with blood after 2–3 days. The patient becomes breathless and toxic. Clinical examination reveals a toxic patient with temperature ranging between 100–105°F, tachycardia, tachypnoea and hypertension in few. Examination of the respiratory system reveals fine crepitations which in the initial stage are confined to the bases but soon become extensive involving bilateral lung fields. Massive haemoptysis may cause asphyxiation and death. Mortality in these cases is very high and it may be as high as 50–70% in cases who report late to the hospital. Chest X-ray in these patients shows bilateral alveolar shadowing which appears to be denser in the mid and lower zones.

clear up within one week without any residual damage.[156]

2.14.5 Other complications

Myocarditis

Cardiac complications are frequent in leptospirosis. They are usually mild and observed as electrocardiographic abnormalities ranging from low voltage complex nonspecific ST and T wave changes, conduction defects and arrhythmias. fibrillations are the most common arrhythmia. Rare but more severe manifestations include cardiac dilatation, cardiac failure and severe arrhythmias from haemorrhagic myocarditis. Sudden death may occur from cardiac failure and arrhythmia. All cardiac abnormalities revert back to normal within 2–3 weeks.[155]

Haemorrhage

Bleeding is a constant feature of leptospirosis and is believed to be due to vascular damage.

It is usually mild in anicteric cases but more common and severe in icteric cases. Bleeding may occur from the respiratory alimentary, renal and genital tract and occasionally into the subarachnoid space and adrenal glands. Death may occur from massive bleeding.[155]



causes of hypovolemia are: i) hypovolemia secondary to vomiting, increased insensible water losses and diminished fluid intake, ii) massive haemorrhage most commonly gastrointestinal, iii) myocardial dysfunction, iv) adrenal haemorrhage, v) widespread vascular injury leading to fluid shifts from intravascular to extravascular fluid spaces and vi) unidentified exogenous endotoxin.[155]

Uveitis

It is a late complication of anterior uveal tract and presents clinically as iritis, iridocyclitis and rarely as chorioretinitis. This may occur in the second week of illness or may be delayed up to 1 year but is more frequent in the first 6 months. Uveitis may be unilateral or bilateral and the course is variable (i.e. acute benign episode, recurrent episode or chronic process).

This ultimate prognosis is good but chronic uveitis may cause blindness from cataract formation and hypopyon in the anterior chamber.[155]

Leptospirosis during pregnancy

The hazards of leptospirosis during pregnancy include intrauterine infection with fetal death and abortion, stillbirth, premature labour and signs of congenital leptospirosis within a week or two of delivery. Leptospirae may be secreted in the milk of lactating mothers, who during the septicemic phase should be regarded as potentially infecting breast fed infants.[155]

rare in cats, and less common in sheep than cattle. The primary reservoir hosts for *Leptospira* serovars are wild mammals, particularly rodents. Reservoir hosts among domestic animals include cattle, pigs, sheep and dogs. The specific reservoir host varies with the serovar and the geographic region. Disease in reservoir hosts is more likely to be asymptomatic, mild or chronic. *Leptospira* infections may be asymptomatic, mild or severe, and acute or chronic. The clinical signs are often related to kidney disease or reproductive dysfunction. Chronically infected animals are asymptomatic.

2.15 Clinical leptospirosis in animals

2.15.1 Acute leptospirosis

Acute leptospirosis is similar in all animals. In its most flagrant form it is manifested by listlessness, loss of appetite, irritability, fever, ruffled fur, red eyes, and sometimes diarrhoea, occurring 3–7 days after infection. There may be signs of haemorrhage and jaundice. Movement is accompanied by a characteristic arching of the back. Recovery may occur from here, or death may supervene. Recovery may be accompanied by weight loss, chronic renal failure and its signs, and delayed death. In producing cattle, there may be disturbance of milk flow and quality. Congenital infection of fetuses in utero follows a similar course, leading to abortion if the fetus dies. Abortion products may be haemorrhagic or jaundiced or both; they may be heavily infected with leptospires and a danger to animal handlers. Stillborn fetuses may likewise

2.15.2 Chronic leptospirosis

Animals which have recovered from acute leptospirosis may develop a carrier condition in which leptospirae grow and may remain in the renal tubules, for periods of days or years. From here they are passed out in the urine. These excretor animals are the main points of distribution for leptospirosis in other animals or people. Leptospirae may also persist in other organs, notably the genital tract. In horses especially, a form of immune iridocyclitis can lead to blindness.[37]

2.15.3 Leptospirosis in Cattle:

Bovine leptospirosis occurs world-wide and is most frequently the result of infection with serovars hardjo (genotype hardjobovis or hardjo-prajitno), pomona, or typhosa. Serovars icterohaemorrhagiae, bratislava, hebdomadis, kremastos, tarassovi, autumnalis, au-sejroe, canicola, and bataviae, zannoni, saxkoebing, and other serovars in the Pyro-Icterohaemorrhagiae, Hebdomadis, and Tarassovi serogroups have also been reported. The most common clinical syndrome associated with acute leptospirosis occurs in cows as a transient fever with a precipitous drop in milk production lasting for two to three days.[37] Acute leptospirosis occurs mainly in calves. The symptoms may include anorexia, conjunctivitis and diarrhea. Severely affected animals may also develop jaundice, hemoglobinuria, anemia, pneumonia, or signs of meningitis such as incoordination, salivation and muscle rigidity. Some calves may die within 3 to 4 days and the survivors can be unthrifty after recovery. The clinical signs vary with the serovar. Infections with serovar hardjo, for example, are not usually associated with her

abortions, decreased fertility or decreased milk yield. Some serovars cause late abortions, stillbirths and increased neonatal mortality. The placenta is retained in 20% of the cows that abort, and infertility may be a sequela.[34] The chronic form of bovine leptospirosis, most commonly due to serovars hardjo and pomona, is associated with fetal infection in pregnant cows presenting as abortion, stillbirth, or birth of premature and weak infected calves.[37] Some serovars can cause sudden agalactia and decreased milk production. The milk may be thick, yellow, and blood-tinged but there is typically little evidence of mammary inflammation. The appearance of the milk usually improves in 4–5 days, and milk production returns to normal after 10–21 days. Jaundice may be seen in severely affected animals.

2.15.4 Leptospirosis in Sheep and goats:

Although the clinical signs have been well described, acute or carrier infections in animals are reported relatively rarely to cause abortion, stillbirth or silent seroconversion and have not assumed the economic dimensions of leptospirosis in cattle and pigs. The main serovars are pomona, grippityphosa, hardjo and bratislava.[37] Leptospirosis in sheep and goats is similar to the disease in cattle.[157] It is characterized by fever, anorexia and, in some animals, jaundice, hemoglobinuria or anemia. Abortion, stillbirths, weak lambs or kids and infertility can also be seen, either with or without clinical signs. Clinical disease is relatively uncommon in sheep.[34]

causing swine leptospirosis include pomona, tarassovi, bratislava, and grippotyphosa. Swine are less commonly infected with serovars sejroe, icterohaemorrhagiae, or canicola. The development of survivors of acute leptospirosis may be unthrifty and the animals may appear sickly. These manifestations are often accompanied by urinary shedding of leptospires.[37] In swine, clinical leptospirosis is most often characterized by reproductive signs including late term abortions, infertility, stillbirths, mummified fetuses, and increased neonatal mortality. Fever, decreased milk production, and jaundice may also be seen. In some infected herds, the only sign of infection may be a transient fever. Subclinical infections are common.

In piglets, there may be fever, anorexia, depression, diarrhea, jaundice, hemoglobinuria, and gastrointestinal disorders, as well as signs of meningitis. Affected piglets may grow more slowly than normal and high mortality rates can be seen in young piglets.[44]

2.15.6 Leptospirosis in Horses:

Many infections in horses are subclinical. Ocular disease is the most common syndrome. During the acute phase, ocular signs may include fever, photophobia, conjunctivitis, chemosis and iritis. Corneal opacity and periodic ophthalmia may be sequelae of acute infections. In the chronic phase, there may be anterior and posterior adhesions of the iris to a turbid vitreous body, cataracts, uveitis and other ocular abnormalities. Although systemic disease is uncommon, severe cases of leptospirosis accompanied by renal, kidney or cardiovascular disease have been described. Two to eight months after

Leptospirosis has also been found to be associated with a number of abortions.[34, 35]

2.15.7 Leptospirosis in Dogs:

The clinical signs and severity of disease are highly variable in dogs. Some infections are asymptomatic or mild, while others are severe or fatal. The initial signs are usually nonspecific and may include fever, depression, anorexia, stiffness, myalgia, shivering and weakness. The mucous membranes are often injected. These symptoms may be followed by signs of kidney disease including anuria, hematuria or increased frequency of urination, vomiting, dehydration and oral ulceration. Abortions, diarrhea, gray diarrhea, coughing, dyspnea, conjunctivitis, weight loss and jaundice may also be seen. Hemorrhagic syndromes occur in some dogs; the mucous membranes may show widespread petechial and ecchymotic hemorrhages and, in later stages of the disease, there may be hemorrhagic gastroenteritis and epistaxis.

Some dogs die peracutely without clinical signs. Chronic kidney disease can be a sequela. Chronic infections may be asymptomatic, or associated with fever of unknown origin, conjunctivitis. Some serovars are more likely to cause certain syndromes. Hemorrhage, anemia and jaundice are typically associated with the serovar *icterohaemorrhagiae*.

Serovar *grippityphosa* tends to cause severe acute kidney failure and/or chronic hepatitis. Dogs infected with serovar *pomona* are often asymptomatic and chronic carriers. Serovar *canicola* often causes chronic interstitial nephritis. Dogs may survive infection with either serovar *canicola* or *icterohaemorrhagiae* and, those that do, often develop profound renal scarring and various levels of chronic renal insufficiency for the remainder of their lives.

prevalence of clinical signs indicates that subclinical infections are relatively common in dogs. These dogs often develop chronic infections, particularly with serovar canicola. They shed large numbers of leptospires in their urine for months to years after infection. Some of these dogs will eventually succumb to chronic renal disease. [37, 41]

2.15.8 Leptospirosis in Wild animals:

Infections are often without any clinical signs in wild animals, including rodents.[34]

2.15.9 Leptospirosis in Sea lions:

Clinical signs reported in seals and sea lions have included depression, polydipsia, abortions and neonatal deaths.[158] Severe interstitial nephritis and gastroenteritis have been reported in infected seals. Hepatitis may not be grossly apparent but the gall bladder may contain inspissated black bile.

2.16 Laboratory Diagnosis

Laboratory participation in the diagnosis is mandatory because the clinical signs and symptoms are not specific in either human or animals. The diagnostic method depends on the samples available and purpose of the testing. Identification of the infecting serovar is of importance both epidemiologically and clinically, since they assist in determining the source of infection. The test can be divided into those that detect bacteria, their antigens or genomic material and those that detect host antibody

and veterinary samples.

2.16.1 Direct demonstration:

Includes microscopy, staining, culture and animal inoculation.

2.16.2 Microscopy:

Leptospires may be visualized in certain clinical material by dark-field microscopy, immunofluorescence or light microscopy after appropriate staining.

From blood leptospires can be seen directly only during first few days. Approximately 10th day of illness leptospires can be demonstrated in urine during leptospiric phase.

2.16.3 Dark ground microscopy (DGM):

Principal of DGM: The principal of darkground microscopy is that the specimen is illuminated only by rays of light that are 'scattered' by objects, e.g. bacteria, of different refractive index from the suspending medium. Light rays enter the objective and reach the eye. In this way, these objects appear brightly against a dark background and peculiar corkscrew motility of the leptospires can be noted.

2.16.4 Demonstration in blood:

Ideally about 5 mL of blood treated with anticoagulant like EDTA is centrifuged at 1000 rpm for 15 min. The supernatant can be examined under 20× objective to observe the typical flexuous motility using dark field or phase contrast. The use of a thin slide is recommended.

Approximately 10^4 leptospire/mL are necessary for one cell per field to be visible in dark-ground microscopy (DGM).[159]

Demonstration in urine: Urine should be centrifuged at 3000 rpm for 10 min and a deposit is examined under dark ground microscope at 20× or 40×.

Advantages of microscopy:

- Dark ground microscopy is very useful to observe leptospire in culture and
- Laboratories, where the facilities for the other tests are not available undertake this technique.

Disadvantages

- Low sensitivity and specificity
- Serum proteins and fibrin strands in blood resemble leptospire.
- Requires technical expertise
- Equivocal results.

2.16.5 Staining:

Many methods for staining such as Congo Red Method, Giemsa stain have been attempted. Silver impregnation method like Fontana staining shows black spirochetes against pale yellow or brown tissue elements. Warthin starry stain is widely used in histological examination. Silver staining methods are considered to be less sensitive and are not recommended as diagnostic procedures.

presumptively. A double layer sandwich method is used with primary sp
antileptospiral antisera and a secondary universal fluorochrome labeled anti-
globulin serum. However, all staining techniques suffer from the same shortcomi
Darkground microscopy, particularly when few leptospires are present in the specin

2.16.6 Isolation of leptospires in culture:

The samples can be inoculated in suitable culture media or in laboratory animals.

Leptospires could be isolated from blood and Cerebrospinal fluid (CSF) o
leptospiiraemic phase and from urine during leptospiuric phase.

Blood culture:

Blood should be taken in around 0.1–0.2 mL (1–5 drops) quantity with all a
precautions and inoculated in to screw capped bottles containing 5–10 mL of me
Usually serum containing media like Korthof's, Stuart's and Fletcher's med
preferred. Leptospires can also survive in conventional as well as newer blood c
media aerobically at 30°C depending on constituents of these media and Palmer
have experimentally proved this in 1984 and again in 2000.[160] Selective r
containing sulfathiazole (5.0 µg/mL), neomycin sulfate (5.0 µg/mL), 5-fluorc
(100.0 µg/mL) and cycloheximide (0.5µg/ mL) may be added to reduce ri
contamination.

Subcultures should be ideally made within 48 hrs to minimize the inhibitory effect
selective agents on leptospires.

Urine and CSF culture:

Samples such as CSF and urine can be inoculated in volumes of 0.5 mL in 5.0 semisolid medium.

Advantages of isolation:

- Definite proof of infection (Koch's postulates)
- Circulating serovars can be assessed
- Local Isolates can be used as antigens in MAT
- Local Isolates can be used in vaccine development

Disadvantages:

- Fastidious organism, requires special medium for isolation
- Leptospire grow slowly. Isolation of leptospire from clinical specimens several days to several weeks.
- The technique is laborious, time consuming and not possible in small laboratories
- Contamination of culture media by other microorganisms or by saprophytic leptospire may occur.
- The successful isolation rate is less due to prior use of antibiotics, impure cleaned glassware or wrong incubation temperature and pH.

leptospire. Around 0.5–1.0mL of inoculum can be injected in intraperitoneally in one of the lower quadrant of abdominal wall. After 3 days, the peritoneal fluid is examined daily for 15 days. If the fluid shows the presence of leptospire, it is inoculated into the culture medium. If the animal dies, the blood collected after mortem should be inoculated in suitable medium. The part of blood collected is allowed to clot and serum is to be screened for antibodies. Liver, spleen or kidney is emulsified in saline & inoculated into suitable medium. Guinea pig or hamster is inoculated intra peritoneally with about 1.0mL of contaminated culture. Leptospire migrate more rapidly than other bacteria from peritoneal cavity and can be recovered from blood taken 20 min to 1 hour later. [75]

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

Identification of leptospiral isolates:

After microscopy and cultural characteristics leptospiral isolate is further identified. A pure leptospiral culture is identified as belonging to a pathogenic or saprophytic species by a variety of tests:

- The ability to infect animals;
- The relative resistance to 8-azaguanine;
- Biochemical reactions: various biochemical reactions like catalase [161], oxidase test demonstration of lipase activity.
- Salt and temperature tolerance;

essential if detailed information on the epidemiology of leptospirosis in par geographical area is to be obtained. For complete identification, a combinat procedures must be used to determine:

- 1) If the isolate is a pathogen or a saprophyte;
- 2) The species of *Leptospira* to which the isolate belongs;
- 3) The serogroup and serovar of the isolate.

The conventional method of identification of leptospiral isolates is based on sero which involves the comparison of surface antigens of isolates with those of ref strains. Strains belonging to *Leptospira* can be differentiated to the serogroup le cross-agglutination reactions. Further differentiation to the serovar level is traditi by cross-agglutination absorption. Although for most isolates this is now being using less time-consuming methods: factor analysis involving major and minor a schemes, monoclonal antibodies (MAbs).

Restriction endonuclease analysis, and various PCR strategies like random am polymeric DNA fingerprinting (RAPD), restriction fragment length polymor (RFLP) are also being used now.

However, genetic-based tests may not always give the same results as the agglutination absorption test.

2.16.7 Susceptibility testing:

Leptospire are susceptible to beta-lactams, macrolids, tetracycline, fluoroquinolon streptomycin.[37] Minimum bactericidal concentration (MBC) is several ord

microbial agents for treatment. Obstacles in the susceptibility testing include:

- Slow growth, which can degrade antibiotic
- Use of media containing serum interferes the test due to protein binding
- Difficulty in quantifying growth accurately
- No standard interpretative criteria.

Whittington et al. has developed a microtitre plate method for testing and used redox indicator to reduce incubation time to 48 hours. The results have been found to be comparable with other susceptibility methods.[163]

Studies have been conducted to study susceptibility of drugs against malaria and leptospirosis, both common in tropics. The only agents identified with the potential to treat both infections other than doxycycline were azithromycin and clindamycin.[164]

2.16.8 Serological diagnosis:

Most cases of leptospirosis are diagnosed by serology. Antibodies are detectable in blood approximately 5–7 days after onset of symptoms. Due to the limitations of bacteriological, especially culture methods, serology is the mainstay of diagnosis. The presence of IgM antibodies in the blood of suspected patient indicates recent infection. Serological methods can also be used for the identification of the infecting serovar.

The serological methods are divided into

1. Genus specific tests-which identify that leptospirosis as the cause of the disease but not the infecting serovar.
2. Serovar specific tests-which identifies specific infecting serovar and helps to

Genus specific test

- Macroscopic Slide Agglutination Test (MSAT),
- Indirect Fluorescent Antibody Test (IFA),
- Indirect Hemagglutination Assay (IHA).
- Counterimmuno electrophoresis (CIEP),
- Complement Fixation Test (CFT),
- Enzyme linked immunosorbent assay (ELISA),
- Microcapsule agglutination test (MCAT),
- Lepto Dri Dot,
- Lepto lateral flow assay
- Latex agglutination test (LAT)
- Lepto Dipstick assay

Serovar/ sero group specific tests-

- Micro capsular agglutination test (MCAT),
- Serovar specific macroscopic agglutination test (MSAT),
- Microscopic agglutination test (MAT),
- Serovar Specific ELISA.

Among all tests Microscopic Agglutination test (MAT) is still considered to be the standard for confirmation of the diagnosis. Usually antibodies (IgM) appear within

similar intervals if necessary.

There may be a titre of antibodies even in case of healthy people in the regions endemic for leptospirosis. In such scenario, a single low titre gives insignificant information regarding infection. Collection of paired sera at certain intervals and the highest titer of later sera will give more appropriate idea about the infecting serovar actually affecting the patient. In case of infection by any serovar, the treatment line does not change. Identification of the serovar has only epidemiological importance.

Brief overview of the various tests used in leptospiral diagnosis is as follows:

Genus specific tests:

Macroscopic slide agglutination tests (MSAT):

The test uses the antigen prepared from saprophytic *L. biflexa* patoc-1 strain. Organisms are grown in suitable medium. After attaining suitable density, organisms are killed by formalin. The centrifuged deposit of killed leptospires is boiled to obtain a creamy white suspension. One drop of serum and 1:10 diluted serum is mixed with 1 drop of antigen on a glass plate. The plate is rotated at 120 rpm for 4–5 minutes and result is read.

4 +—clearly defined clumps against a clear background.

3 +—obvious agglutination but suspension not completely clear.

2 +—Fine clumps with 25% agglutination,

sensitivity of the test. Brandao et al. in 1998 compared MSAT with IgM ELISA and MAT. He concluded that MSAT and ELISA are more sensitive as initial screening tests, MSAT being easier than ELISA.[165]

Indirect Immunofluorescence Antibody test (IFA):

It is a reliable test where the facility of fluorescent microscopy is available. It is used in diagnosis of leptospires in tissue sections. Torton described indirect immunofluorescence using genus specific patoc strain and described its advantage over MAT. A study compared IFA test with MAT and showed that IFA test was more sensitive and specific for the initial diagnosis of leptospirosis. It could replace the complicated and less sensitive microscopic agglutination assays.[166]

Indirect Hemagglutination Assay (IHA):

Substances which adsorb on erythrocytes have been extracted from various organisms—these are known as erythrocyte sensitizing substances [41]. Two serological reactions can be demonstrated when ESS sensitizing cells are mixed with sera containing the homologous antibodies: haemagglutination (HA) or sensitized erythrocyte agglutination (SEA) or hemolysis or sensitized erythrocyte lysis (SEL). Chan and McComb were the first to report erythrocyte-sensitizing substance, which is both reactive against leptospiral antibodies with higher specificity.[167] Several experiments later showed this test to be cumbersome and less sensitive [75] (Turner). In 1957 Turner et al. reported in detail preparation, standardization and stabilization of ESS extracted

1975 Sulzer et al. showed that the indirect hemagglutination test developed at CDC had a sensitivity of 92% as compared to 69% for the presumptive slide agglutination test. The specificity was 95% for hemagglutination test as compared to 83% for the slide test. In 1998, Levette et al. reported sensitivity of IHA for detection of acute leptospirosis as 100%, the positive predictive value 95% and negative predictive value 100%.[170] In 2001 Levette et al. compared IHA with IgM Dipstick assay. The sensitivity was reported to be 92.2% and specificity 94.4%, positive predictive value 95.9% and negative predictive value 92.7%.[171] Based on these reports IHA was suggested as a suitable test in a diagnostic laboratory. The test has again been evaluated at Hawaii in year 2002 and low sensitivity in contrast to earlier reports has been reported.[172]

Counter immuno electrophoresis (CIEP):

Its application in the detection of leptospiral antibodies for the serodiagnosis of leptospirosis has been reported earlier by Terpstra et al. Myers comparatively studied CIEP with MAT. The agreement between the CIEP and MAT with sera from patients with suspected cases of leptospirosis and a group of healthy individuals was 97.9%.

Complement fixation test (CFT):

Complement fixation test can be used as a screening test. The antigen is derived from *L.biflexa*, patoc-1 strain. Results can be read objectively and quantitatively with laboratory instruments and large quantity of sera can be screened. The limitations include anti complementary activity of sera and short life of reagents. Turner et al. have re-

CFTs of human sera for Weil's disease. They concluded that it was a trustworthy diagnostic aid.[174]

Enzyme linked immunosorbent assay (ELISA):

Enzyme linked immunosorbent assay involves detection of antigen or antibody by enzyme linked anti-human antibody and suitable substrate. This method can detect approximately 10^5 leptospires/mL.[159] Each ELISA uses stable reagents and forms the basis of bedside tests, which are read visually. The use of computer based auto readers and appropriate controls improve the reproducibility and predictive value of the test. Different types of antigens have been prepared by different methods. They are usually genus specific. Commercial kits use synthetic antigen, using specific immunogens of the genus *Leptospira*. Using ELISA one can detect IgM as well as IgG antibodies using anti-human IgM and IgG respectively.

Cumberland et al. in 1999 assessed the efficacy of IgM ELISA and MAT. The sensitivity of IgM detection by ELISA was 52% in first acute phase specimen, increasing to 85% and 93% in second acute phase and convalescent specimens. The sensitivity of MAT was only 30% in the acute phase specimen. Hence, ELISA method was reported to be more sensitive.[175]

The ELISA methods have been modified further. The IgM specific dot ELISA was developed, in which polyvalent leptospiral antigen is dotted onto, nitrocellulose on microtitre tray plates allowing use of smaller volume of reagents.[176] Marud Desilva and colleagues evaluated diagnosis of leptospirosis by Dot ELISA for

Serovar specific tests

Serovar specific macroscopic agglutination test (MSAT):

A battery of serovar antigens prevalent in the area can be used in it. In Germany macroscopic slide agglutination test 12 antigens were originally proposed and a supplementary antigen was suggested.

Microcapsule agglutination test (MCAT):

Synthetic polymer carriers are sensitized with mixed antigens of sonicated leptospiral antibodies. Arimitsu et al. evaluated a simple MCAT in China using synthetic polymer instead of red blood cells. He compared MCAT with MAT and reported a relatively genus specific tendency with a 4–32 fold higher sensitivity. The sensitized microcapsule antigens were stable for at least 1 year. They proposed MCAT as a screening test for the diagnosis of leptospirosis.[178] An international multi-centre evaluation of one point microcapsule agglutination test was done and reported in the World Health Organization (WHO) bulletin year 1994. The test was found to be more sensitive than MAT and IgM ELISA in early acute phase samples. However the test failed to detect infections caused by some serovars.[179] Sehgal et al. evaluated MCAT and reported overall sensitivity and specificity of 84.7% and 87.0% respectively in comparison to MAT.[180]

Lepto Dri-Dot:

It is a new card agglutination test developed by the Dutch Royal Tropical Institute. This assay helps in rapid diagnosis. It was evaluated in field conditions of the Andaman Islands and was shown to have a sensitivity of 67.6% (50/74) and a specificity of 85.5% (33/50) during week 1. During weeks 2–4 the values increased to 85.5% (47/55) and 89.5% (40/50) respectively. Both Dri Dot and ELISA showed good agreement with the standard diagnostic criteria after the first week of illness and the overall concordance of the test was 89.5%. Adding to the advantage is the fact that the test does not require special storage or sophisticated equipment and can be performed by relatively low skilled personnel.

Lepto lateral flow assay:

The Royal Tropical Institute has recently developed this test. It is based on the binding of specific IgM antibodies to the broadly reactive heat extracted antigen prepared from non-pathogenic *Paratyphus* 1 strain. IgM antibodies bound to the broadly reactive antigen are detected with an anti-human IgM gold conjugate contained within the test device. Smiths et al. in 2002 evaluated this test at the Andaman Islands, and the results were compared with the standard criteria. Lepto lateral flow had a sensitivity of 52.9% in the first week of illness and 86.0% during 2nd and 4th week. The corresponding specificities were 93.6% and 89.4% respectively. All indices of validity and utility of lateral flow were similar to IgM ELISA and Lepto dipstick. The test can be performed at the bedside of the patient and whole blood can be used for testing. [181] Smiths et al. in 2001 have shown that this device has a sensitivity 85.8% and specificity 93.6%, which compares well

Latex agglutination test (LAT):

This test depends on sensitization of commercially available latex particles with a leptospiral antigen. Antigen is prepared from *L. biflexa* patoc 1 strain.

Smith et al. in year 2000 evaluated LAT consisting of individually wrapped agglutination cards containing a stable, dried detection reagent. During the first 10 days of illness sensitivity was 72.3% and at a later stage it was 88.2%. The specificity was 93.3% and 89.8% respectively.[183] Hull-Jackson et al. evaluated a commercial LAT for serological diagnosis of leptospirosis. They reported that LAT was more sensitive for acute phase samples than MAT. However they emphasized the need to evaluate these assays for use in different populations. The other advantage of the test include limited amount of biomedical waste generation and prolonged shelf life at varied temperatures from 2°C to 45°C. [184] Dey et al. have developed a rapid recombinant antigen LipL32 based LAT. LipL32 is an immunodominant antigen. Sensitivity and specificity of this test with MAT was evaluated and it is 93.93% and 96.72% respectively on human serum samples.[185]

Lepto Dipstick assay:

This assay also aims at detection of IgM antibodies in patient's sera. Smits et al. conducted multi center evaluation of dipstick assay' internationally and published reports in year 1999. The results of the dipstick assays 'were concordant with that of IgM ELISA.[186] However, according to some reports the test is cumbersome and takes approximately 3 hrs to give a colored band and is not recommend and is unsuitable in view of more rapid and better tests now being available.[187]

Microscopic agglutination test (MAT):

This is the reference method for serological diagnosis of leptospirosis. Patient's serum is reacted with antigen suspensions of leptospiral serovars. After incubation, the serum-antigen mixtures are examined microscopically for agglutination and end titre is determined. Formerly the method was known as agglutination lysis test due to formation of lysis balls. However later it was proved to be a misconception. Actually these proved to be the tightly agglutinated clumps of leptospires containing live cells and debris. [188]

Microscopic agglutination test can be performed using suspension of live culture or cultured killed by formaldehyde.

MAT using live cultures:

Living late logarithmic or early stationary growth phase cultures of leptospires grown for 4–14 days in liquid medium at 28–30°C are used as antigen suspension. Culture clumps of leptospires or precipitates may be filtered or centrifuged lightly, so long as final density is not below the preferred density of $1-2 \times 10^8$ leptospires/mL.

For diagnostic tests, it is advisable to use locally isolated or locally dominant antigenically authenticated strains.

MAT using killed cultures:

Cultures killed by formaldehyde can be used for MAT. They are safer to use than live cultures, can be used for a longer time and the clumps of agglutinated killed leptospires

Performance of the MAT test: The serum to be tested is serially diluted starting from 1:10 and later using 2-fold dilutions in a microtitre plate or in tubes. Equal volume of antigen suspension is added to each dilution and the mixture is then incubated at 37°C for 2 hours. After 2 hours, the suspension in each well is examined microscopically for agglutination. Agglutination is noted by observing clumps and the density of residual, unclumped leptospires. The end point is the highest tube or well in which 50% agglutination is recorded, measured by comparison of the density of agglutinated leptospires with a control suspension in which 1 volume of diluent was incubated with 1 volume of antigen suspension, without serum.

There are few limitations to the MAT although it is a recommended gold standard.

- A preparation of MAT requires meticulous maintenance of several strains in the laboratory. This needs preparation of large amount of culture media, regular subcultures and quality control. The cultures carry continuous risk of contamination by other strains.
- A single serum sample does not provide any idea about recent or past infection. MAT detects both IgM and IgA antibodies, so the collection of paired sera is required. A rising titre about fourfold should be demonstrated. Thus MAT has limited use in the early diagnosis.
- Standardization of the test is a problem as the age of culture and density of antigen suspension affect the result.

observer variation.

The advantages of MAT are:

- It indicates the infecting serovar.
- Gives an indication of seroprevalence of the disease in a particular population.

Interpretation of diagnostic MAT:

Interpretation of MAT is complicated by high degrees of cross-reactions that occur between different sero groups, especially in acute phase samples. Sometimes, parareactions, in which the highest titers are detected to a serogroup unrelated to the infecting one, are also observed. The broad cross reactivity in the acute phase followed by relative serogroup specificity in the convalescent phase samples is due to detection of both IgA and IgM antibodies in MAT, and the presence of several common antigens among leptospires. Hence, for the definitive diagnosis, paired sera are required. A four-fold or greater rise in titre between paired sera confirms the diagnosis. The interval between samples can vary depending upon delay between onset of symptoms and presentation to the patient. Delay in seroconversion may increase the interval between samples. A recent infection can be suggested by a single elevated titre in association with an acute compatible illness. Such a titre is dependent upon the baseline titre of population i.e. seroprevalence. According to CDC case definition 1997, a titre of 200 is used to define a probable case with a clinically compatible illness. In areas where leptospirosis is endemic, a single titre of 800 in symptomatic patient is indicative of leptospirosis, Titre as high as 1600

serogroup. In such cases, serological diagnosis is complicated further by the anamnestic response in which first rise in antibody titre is usually directed against the initial serovar or serovar from the previous exposure. This further complicates the diagnosis. MAT however seems to be the most appropriate test to employ in epidemiological surveys. It can be applied to sera from any animal species and the range of antigen can be expanded or decreased as required. Though the isolation of the infecting serovar is the best for prevalence studies; MAT can still provide ideas about serogroups present in general population.

2.17 Molecular methods for laboratory diagnosis

Leptospiral DNA has been detected in clinical material by dot-blotting and Southern hybridization. Recombinant probe specific for pathogenic serovars was prepared for serovar *lai*.

However, the sensitivity of ^{32}P -labeled probes was less than that of PCR. With the advent of PCR, probes have not been used extensively for diagnosis.

PCR method involves *in vitro* amplification of target DNA sequence, brought about by thermostable DNA polymerase (Taq). A pair of short DNA fragments, known as primers, is used for specific amplification of DNA fragments.

Polymerase chain reaction enables rapid detection of very small amount of organism in the given sample. The method includes:

- Denaturation at 94°C: Separation of single DNA (double stranded) to 2 single stranded molecules.
- Annealing at 54°C: The primer binds to template DNA to which, polymerase

of template by adding bases complimentary to the template at 3' end.

Thus PCR amplifies a single gene into several copies after many cycles. Amplified fragments can be visualized on ethidium bromide stained agarose gel with transilluminator. In Real Time PCR, target amplification and detection steps simultaneously in the same tube and the PCR product is detected as it is produced using fluorescent dyes, which preferentially bind to double stranded DNA. Several primer pairs for the detection of leptospires have been described.

The primers described by Merien et al. amplify a 331-bp fragment of 16 s-RNA gene in both pathogenic and non-pathogenic leptospires, so in case of contamination of clinical samples, the incidence of false-positive reaction increases.[190] While Gravekamp et al. recommends the use of two primer pairs for the detection of all pathogenic serotypes [191]

From many clinical samples, leptospiral DNA has been isolated. Leptospiral DNA has been amplified from serum, urine, aqueous humor, CSF.[9]

More recently a real time PCR has been developed by Smythe et al. using TaqMan chemistry which targeted an 87 bp section of 16 s rRNA gene of *Leptospira* species. Levette et al. have reported the development of real-time assay using SYBR[®] chemistry, in which the target is the gene for the major outer membrane protein L which appears to be an important virulence factor, confined to the pathogenic strains of all *Leptospira* species. [193]

- Rapid detection in a single specimen.
- Detection of very few organisms.
e.g. real time PCR assay detect as few as 2 cells in serum and 10 cells in u
5 hrs approximately.
- Quantitative monitoring of leptospiral cells possible. Possible role in mon
treatment efficacy has been proposed.[192]

Disadvantages

- The procedure is expensive and complicated
- Contamination of test sample with small quantities of DNA from the
experiment may lead to false positive results. No identification of inf
Serovar.

For identification of infecting serovars, molecular typing methods need to get appli

The available methods include:

- Restriction enzyme analysis (REA)
- Ribotyping method
- Random amplified polymeric DNA fingerprinting- (RAPD)
- Pulsed field gel Electrophoresis (PFGE)
- Restriction fragment length polymorphism (RFLP)
- Single strand conformation analysis (SSCP)
- DNA sequencing
- Denaturant gradient gel electrophoresis (DGGE)

certain disadvantages that include requirement of high quality of pure DNA discriminatory power, low reproducibility, ambiguous interpretation of data and problems associated with transfer of data between different laboratories. Ahmed et al. concluded that Multi locus sequence typing (MLST) has advantage over other methods for leptospires include reproducibility, robustness, consistency and portability.[194]

2.18 Measures for control and prevention of leptospirosis:

2.18.1 Treatment

Various antimicrobial drugs, including penicillin and tetracyclines, show antileptospirosis activity. Penicillin (e.g. 6 million units daily intravenously) is the drug of choice in the treatment of leptospirosis and is especially effective if started within first four days of illness. Jarisch-Herxheimer reactions may occur. Total duration of therapy should be 10-14 days. Amoxycillin and erythromycin have also been found effective in severe leptospirosis. Patient should be observed for evidence of renal failure, and treated, if necessary, with hemodialysis. Patients with Weil's disease having hemorrhagic manifestation may require whole blood or platelet transfusion. Patients with multi organ failure require to be observed in intensive care unit.

Besides penicillin, doxycycline in a dosage of 100 mg twice daily for 7 days is effective in treatment of mild and moderate leptospirosis. Effective prophylaxis consists of doxycycline, 200 mg orally once weekly, during the risk of exposure.[49]

transmission.[37] In different epidemiological settings, different animal species could be the primary source of infection. Risk factor analysis highlights significant association of leptospirosis with possible carrier animals viz., cats and rats. While in the Andaman archipelago of the Andaman & Nicobar Islands, cattle were found to be associated with leptospiral seropositivity. Instances where there is absence of statistical evidence of association between contact with an animal species and human leptospirosis, but if a significant carrier state is detected in animals, it could be taken as a potential source of infection to humans. However, as seropositivity is not a reliable indicator of carrier state, demonstration of excretion of leptospires in the urine by bacteriological or molecular tools are required. Several strategies have been described to reduce the load of carriers in domestic animals.

Risk group population (farmers, sewage workers, canal workers, dairy workers etc.) should be encouraged to use personal protection equipments such as gloves, boots, masks, protective goggles (while milking cows) etc. Some safe practices in these high risk groups such as wearing protective clothing in hazardous tasks like clearing of drains, de-weeding/de-silting operations, working in sewers or during flood relief activities, washing or showering after exposure to contaminated soil or water, hygienic practice while handling pets and domestic animals and washing of hands after each contact can reduce risk of exposure.[116]

Measures targeting human beings include chemoprophylaxis and vaccination. Chemoprophylaxis with doxycycline has been tried in soldiers from non-endemic areas visiting endemic areas and was found to be almost 100% effective, while it gave only 54% protection. Thus, chemoprophylaxis can be used in outbreak situations or in

characteristics of a community that faces the problem of leptospirosis is the essential prerequisite for devising an effective and acceptable intervention strategy.[5]

2.18.3 Eradication

Although absolute eradication of leptospirosis is a very difficult task, proper prevention and control methods could greatly reduce the incidence of this disease in both man and domestic animals. However, because of the development of diagnostic methods and pharmaceutical elimination of the carrier state, it is now reasonable to attempt eradication of the disease from individual herds, and possibly from areas. The principal hazard of such a scheme is the introduction of carrier animals of any species, or by reintroduction of the infection by rodents or other wildlife. It is because of this hazard that eradication programmes aim at containment rather than eradication. In these circumstances where only sporadic cases occur, it might be more profitable to attempt to dispose of reactors or treat them to ensure that they no longer act as carriers. Detection and elimination of carrier animals are difficult tasks. Positive reactors to the MAT may not shed leptospires in urine and to determine their status as carriers, repeated examination of their urine by culture and guinea-pig or hamster inoculation is necessary. For practical purposes, suspicious and positive reactors to the serum test should be considered as carriers and culled or treated unless examination of the urine can be carried out.

In cattle herds the eradication might seem simpler because of the easier identification of carriers. However, an eradication programme is rarely if ever adopted. Some consideration must be given to the bulls because if they are infected they should not be used naturally for artificial insemination, even though the standard concentration of penicillin and streptomycin in the semen diluent is sufficient to ensure that no spread occurs.[43, 194]

pass a serological test on 2 occasions at least 2 weeks apart before allowing them to enter the herd. Urine examination for leptospires should be carried out if practicable. Control of shipment of domestic animals across country and international is also an important factor in the eradication of leptospirosis. It is strongly recommended that each country or region planning to import domestic animals demand certificates of leptospiral free herds with shipment.

Hygienic methods for prevention of direct and indirect human contact with animals have often been recommended. If the immediate source of infection is identifiable, such as a form of yards, marshes and damp calf pens, every attempt must be made to avoid contact between animals and infected surroundings. Damp areas should be drained or fenced and pens disinfected after use by infected animals. The possibility that rats and other wild animals may act as a source of infection suggests that contact between them and farm animals should be controlled.[5]

2.18.4 Vaccination:

Vaccines have been developed for use in man.[196] Existence of a large number of serovars makes it difficult to develop a universally effective vaccine. Leptospires have evolved ways to evade host immune response.[197] Because of the ability of pathogenic leptospires to translocate between mammalian cells quickly and reach blood stream, their ability to enter and initiate programmed cell death in kidney fibroblasts rapidly,[144] it is difficult for the immunologists to develop effective and safe leptospirosis vaccines. Currently the molecular and cellular studies on leptospirosis

proteins have been constructed and tested for their usefulness as vaccine candidates. Amongst these, OMPs, lipoproteins and virulence factors have generated considerable interest. *Leptospira* external membrane protein LipL32 cloned and expressed in mycobacterial vectors have been found to be protective to hamsters. In 2002 a 13 kDa immunoreactive leptospiral immunoglobulin-like protein (LigA) was described (Palaniappan et al. 2002). LigA as well as another immunoglobulin-like protein were found to be immunogenic. It has been shown that LigA DNA vaccine provides significant protection against leptospirosis in hamsters. Inactivated and attenuated vaccines for leptospirosis have been described for more than 50 years. These vaccines have been tested in cattle and some of these are currently being used. Some of these vaccines have been tested in humans as well. At present, the only leptospirosis vaccine licensed for use in humans is being produced in Cuba. However, there is still an interest in inactivated and attenuated vaccines. Recently the focus is on newer vaccines such as DNA vaccines. At least two DNA vaccines, one encoding haemolysis-associated protein (Hap1) and the other endoflagella gene (flaB2) have been tested. These DNA vaccines require more extensive investigations.[5]

*Aims
&
Objective*

leptospirosis has now been recognized as an emerging infection. Human leptospirosis epidemic has occurred increasingly and continuously from many years in India. This can be used to hypothesize that the source of leptospirosis infection was continuous and circulate in the outbreak areas. The source of infection could have been carrier animals that shed *Leptospira* spp. or the environment that may be contaminated with leptospires. Therefore, the objective of this study was to determine the possible sources of leptospirosis infection including carrier animals (i.e. dogs and rodents).

Risk factors for leptospirosis include living in rural areas and the epidemiology of human leptospirosis reflects the ecologic relationship between humans and chronically infected mammalian reservoir hosts. Three study epidemiologic patterns of leptospirosis were originally defined by Faine et al. The first pattern is a “farming type” that occurs in temperate regions, due to direct contact with animals through exposure to a large number of serovars that infect cattle and pigs. The second pattern is an “urban type” which comprises rodent borne infection in the urban environment. The third pattern is a “tropical type” that occurs in humid, wet areas, from exposure to a larger number of serovars infecting a diversity of reservoir animals including rodents and farm animals. Recently, those considered at greater risk of contracting leptospirosis include farmers, pig farmers, butchers, agricultural workers and sewer workers along with people who participate in or supervise water-based recreational activities such as canoeing, swimming and sailing. The data generated during study of different risk groups will be helpful for applying preventive measures focusing a particular risk group.

After the 26 July 2005 flood in Mumbai, the disease is more prevalent in and around the Mumbai region. However, true incidence and prevalence of leptospirosis in Mumbai

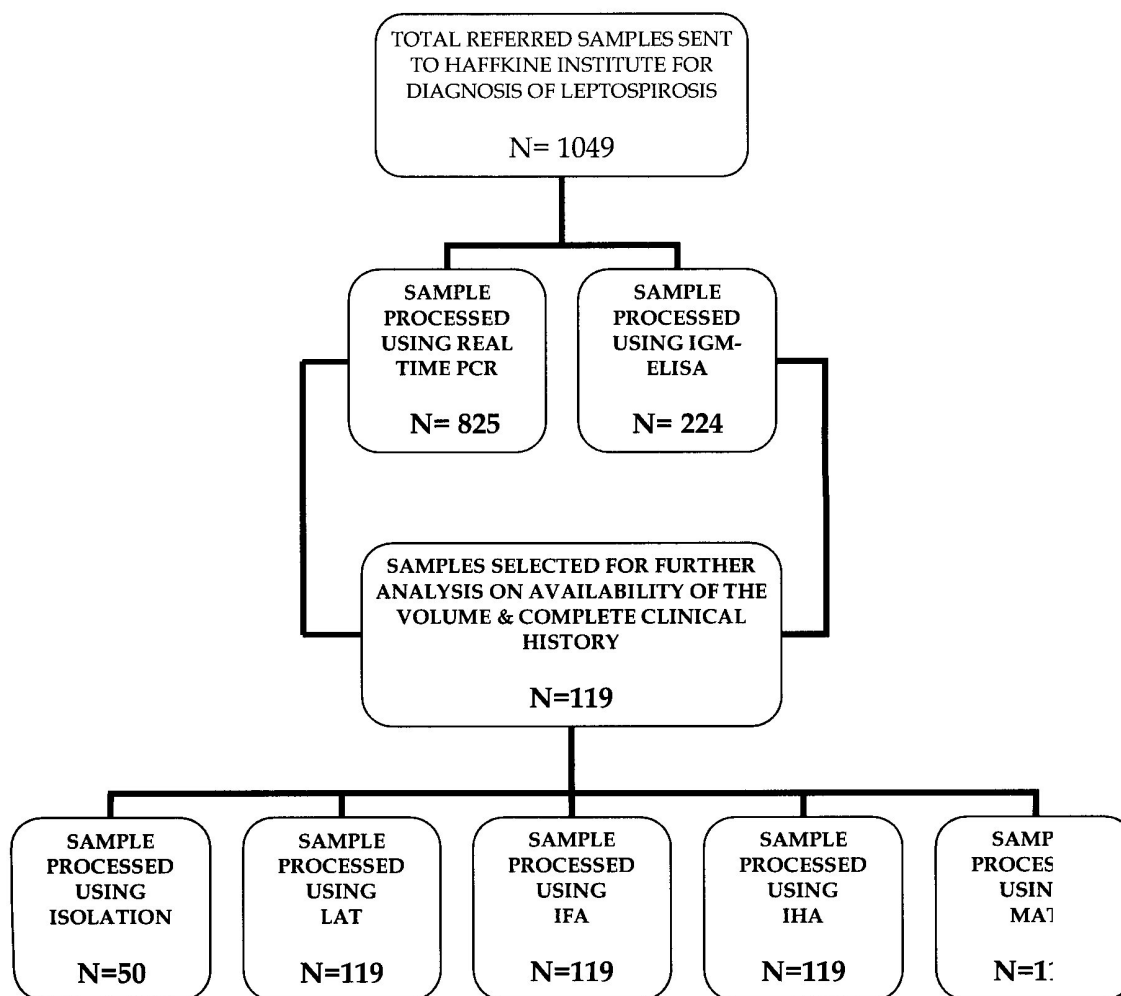
serovar in the study area. The disease has also been a diagnostic enigma for microbiological laboratories across the country. Since isolation rate of the causative organism from clinical specimens is low due to prior indiscriminate use of antibiotics and also due to difficult and expensive isolation techniques, currently, serological techniques remain the cornerstone of diagnostics. Various serological tests like Latex Agglutination Test, ELISA, Immunofluorescence Antibody Test (IFA), MAT and Indirect Hemagglutination Test (IHA) have been used. Microscopic Agglutination Test is considered to be the standard test; however, routine laboratories rarely perform it, since it requires considerable laboratory infrastructure, skilled technicians and is extremely time-consuming and intensive. Many serological tests like Dipstick and IgM ELISA are commercially available. Newer methods such as PCR and Real Time PCR are also proving their importance.

Hence, in our study, we compared different conventional and modern diagnostic tools for diagnosis of leptospirosis in human and animals. This will provide several promising approaches to rapid diagnosis of leptospirosis in the future.

- To study different risk groups, risk factors and sources of infection in the presence of leptospirosis.
- To study the prevalence of leptospirosis in humans and animals.
- Characterization of *leptospira* isolates to species level.
- Assessment of efficacy of different diagnostic tools used for diagnosis of leptospirosis.

*Materials
&
Method*

Figure 7: Schematic flow diagram of human study subject categorization and leptospirosis testing.



Where,

LAT=Latex agglutination test.

IFA=Indirect Immunofluorescence antibody test

IHA= Indirect hemagglutination assay.

MAT=Microscopic agglutination test,

1. BLOOD SAMPLES:

A: Human samples:

During 2006–2008, a total number of 1049 referred samples from the patients suspected of leptospirosis (as per WHO guidelines for diagnosis of Leptospirosis)[74] were sent to the Nodal Laboratories for Re-emerging Diseases (viz. Leptospirosis, Dengue, Malaria etc.) in Department of Zoonosis at Haffkine Institute, Mumbai from various hospitals and around Mumbai region. Out of 1049 samples, as per the request of the referring doctors, Real-Time PCR was carried out on 825 samples and IgM ELISA was performed on 224 samples. Duly filled proforma with respect to demographic characteristics and clinical symptoms were sent along with the samples.

A total of 119 coded samples were selected for further analysis from 1049 samples on the basis of maximum volume of the samples available (as most of the samples sent to the centre were less in volume) and availability of complete clinical history of the patients.

Controls

Ten blood samples were collected from healthy individuals to serve as negative controls. All of them confirmed to be negative for anti-*leptospira* antibodies by an IgM-ELISA for leptospirosis. These samples were used as negative control for some of the diagnostic assays. Informed consent was obtained from the control group.

Due clearances for studies on human as well as animal samples were obtained from the Institutional Ethical Committees of Haffkine Institute for Training, Research and T. Mumbai.

stored at -20°C till further use. Whole blood was used for inoculation into EMJH media for isolation of organism.

B: Animal samples:

i Rodents:

To study the prevalence of leptospirosis in rodents, live rodents were trapped by rat catchers of Brihan Mumbai Municipal Corporation (BMC) and then transported to the Municipal rat destruction establishment (MRDE) of Haffkine Institute.

All these rodents were characterized as per their morphological characteristics and distinguishing features. The rodents that were found in our study are as follows:

A. *Rattus rattus*: (Common house rat)

- i. Normally found near the human dwelling places. It is cosmopolitan, occurs practically all over India in both wild and commensal forms. Burrows in walls and roofs, mostly arboreal.
- ii. It is medium size rat with weight ranging between 200 to 280 g. The body is slender shaped, elongated. Snout is narrow, long, pointed and fleshy. Eyes are well developed and large.
- iii. Eyes well developed, large enough to cover the eyes when folded.
- iv. Total tail length is longer than total length of head and body together. Ringed clear on tail and range between 260–290.
- v. Fur is smooth and bicolored i.e. darker or shiny black on back and lighter on sides.

B *Rattus norvegicus*: (Norway rat or brown rat)

- i. Comes under peridomestic forms of rats, as it prefers living away from human dwellings.
- ii. It has complicated and net like burrows, mostly found in sewer drains, and open grounds near residential areas.
- iii. Body is cylindrical, elongated with large and sharp snout and blunt nose. Body weight ranges between 300–450 g.
- iv. Eyes and ears shorter as compared to *R. rattus*, ears densely covered with hairs.
- v. Length of tail is shorter than total length of body and head. The tail does not possess rings but has matt scales.
- vi. Fur is coarse, thick and bicolored, deep brown on head and abdominal regions.
- vii. Females have 6 pairs of mammary gland.

C *Bandicoota bengalensis*:

- i. This species is a serious pest of agriculture and stored grains and has an important role in transmission of diseases to humans and its live stock. Likes to live in mixed type multi-channeled burrows, both commensal and wild in habitat.
- ii. Eyes and ears are short as compared to its body.
- iii. Tail length shorter than total length of the head and body together. Rings that are not clear are uniform ranging between 200–250.
- iv. Females have 8–9 pairs of mammary glands.
- v. Fur is spinous, black and coarse.

The rodents were taken out of the wonder traps identified according to morphological characteristics as mentioned above and noted. The rodents were placed in desiccators in which cotton soaked in anesthetic ether was added and the desiccators were placed on top.

In this study, 60 rodents were included. Rodents were caught alive from different parts from in and around Mumbai by rodent handlers of the Brihan Mumbai Municipal Corporation (BMC) and were brought to Municipal Rat Destruction Establishment (MRDE) at Haffkine Institute.

Blood collection:

The anesthetized rodents were placed on flat surface and cardiac puncture was performed using 21-gauge needle of 1 inch length. For this needle was inserted from the base of sternum and after penetrating around 1.0 to 1.5 cm straight inside, it was diverted towards the heart at 45° angle. At a point the heartbeat can be felt on the tip of the needle and aspirate the piston slightly as shown in Figure 8. The blood so obtained was collected in sterile tubes. Serum was separated from the plain bulb, aliquoted and stored at -20°C till further use. Whole blood was used for inoculation into EMJH medium for isolation of organism.



Dogs:

Prevalence of leptospirosis was also carried out in dogs. Blood samples were collected from 40 dogs brought for the treatment to Bombay Veterinary College, Parel, Mumbai with the symptoms of anorexia, fever, vomiting, dehydration and icterus.

Blood collection:

A non-slip mat can help hold the dog steady. Animals should be acclimatized to restraint before being used in experiments. Dogs were either be restrained manually or using a sling with help of the staff. Blood sample was collected from the cephalic vein which is superficial and easily accessible. The technique is quick and appropriate for taking small blood samples. Blood is collected from the forelimb, starting distally (near the paw) and working proximally (away from

sampling site was taken into consideration while sample collection. The blood so obtained was collected in sterile tubes. Serum was separated from the plain bulb, aliquoted and stored at -20°C till further use. Whole blood was used for inoculation into EMJH medium for isolation of organism.

Figure 9: Collection of Blood Sample from Dog



URINE SAMPLES:

i. Rodents

A total of 12 urine samples were collected from rodents. Urine was withdrawn aseptically using syringe and needle. Urine samples were diluted using Phosphate Buffered Saline for inoculation into medium for isolation of organism. The remaining urine samples were preserved using formalin for DNA extraction.

A total of 6 urine samples were collected from dogs. Urine was withdrawn aseptically using catheters with the help of veterinarians. Urine samples were diluted using Phosphate Buffered Saline for inoculation into medium for isolation of organism. The remaining urine samples were preserved using formalin for DNA extraction.

5.2 METHODS:

5.2.1 Isolation of organism:

Glassware used for isolation of organism was washed with water, distilled water, dried, further autoclaved to avoid contamination and acidity which will cause destruction of leptospire.

Culture media:

The culture medium used for isolation of the organism was the EMJH (Ellinghausen McCullough-Johnson-Harris) medium which was modified by the addition of serum.

To make the medium selective, 5-fluorouracil was added at a concentration of 100 µg/ml of medium.

Collection of the Rabbit serum:

Under all aseptic precautions, rabbit blood was collected from the central vein of ear plain bulb.

The serum was separated by centrifugation and stored at 4°C.

having a pore size of 0.22 μm diameter.

The serum was added to sterile EMJH medium in 2–10% concentration.

Blood culture:

One to two drops of the blood/diluted urine was added aseptically to 5 mL of medium.

The medium was kept in incubator at 37°C for approximately 48 hours.

Thereafter it was kept at room temperature and observed daily for turbidity, growth

Urine culture:

Urine samples were diluted aseptically in sterile test tube using equal volume of phosphate buffer saline solution (pH 7.2).

Four to five drops of diluted urine samples were then added aseptically to 5 mL of medium.

The medium was kept in incubator at 37°C for approximately 48 hours.

Thereafter it was kept at room temperature and observed daily for turbidity, growth

Culture examination:

All the cultures were observed under the Dark field microscope. Microscopic examination was done every 3rd, 7th and 10th day of inoculation.

Negative cultures were not discarded till 2 months of inoculation.

5–10 µl of the culture was taken on a thin glass slide (1 mm thickness).

A thin cover slip (22 × 22 mm) was placed on the preparation.

The preparation was observed under 20× for the typical motility of the *leptospira*.

The presence of leptospires in the culture was further confirmed by DNA extraction performing Real time PCR as discussed further.

Fontana's staining:

To confirm the presence of organism in the culture Fontana's staining method was used.

All stock solutions were prepared as mentioned in appendix

1. A smear was prepared from culture on the glass slide and fixed with fixative
2. The slide was washed with alcohol and dried by heating gently on a Bunsen burner.
3. Mordant was poured on the slide and heated. The slide was washed again with distilled water and dried.
4. Finally slide was treated with ammoniated silver nitrate till film turned brown on heating.
5. Slide was washed with distilled water and mounted in paraffin and observed under oil immersion lens.

Leptospira appears dark brown colored against light pink background.[198]

5.2.2 Latex Agglutination Test:

Latex Agglutination Test kit was graciously provided by Regional Medical Research Centre (RMRC), Port Blair, Andaman and Nicobar islands. The kit was indigenously developed by RMRC.

Principle:

A latex agglutination assay is a rapid test for the detection of antibodies to leptospirosis using latex beads coated with the *leptospira* antigen. The test involves direct screening of leptospiral antibodies in serum samples by simple visual identification.

Procedure:

The test was performed by placing 5 µL of serum sample on a glass slide. Subsequently, the serum was mixed with 10 µL of coated latex particles. The slide was rotated for 2 min and read for the aggregation of the latex particles on the scale 0 to 3+.

Positive and negative controls were included with every batch tested.[199]

5.2.3 *Leptospira* IgM ELISA (Kit based) :

ELISA test appears to be able to detect anti-leptospiral antibodies somewhat earlier than MAT, beginning around 5 days after symptoms start.

To detect current infection, IgM antibodies were detected from the serum samples. Rheumatoid factors are auto-antibodies, mainly of IgM class. The presence of non-specific IgM antibodies (RA Factor) can lead to false-positive results in the IgM ELISA. Therefore, it is necessary to pre-treat samples with RA factor absorbance prior to detection.

The test was performed according to the manufacturer's instructions.

Microtitre plates are coated with antigens. This constitutes the solid phase. Sample is added to the plates and any antibodies specific for the antigen present will bind to the solid phase.

After removal of unbound material, anti-human IgM conjugated to an enzyme is allowed to react with the immune complex. After removal of excess conjugate by washing, an appropriate substrate is added, with which the conjugated enzyme reacts producing a colored derivative of the substrate. The color intensity is proportional to the level of specific antibody bound and can be quantified photometrically.[200]

Assay procedure:

1. Patient sera were diluted in the ratio of 1:40 using dilution buffer.
2. To the 100 μ L of diluted serum, 40 μ L RF (Rheumatoid Factor) absorbent was added to remove rheumatoid factor and incubated for 5 min. at room temperature (RT).
3. All 140 μ L was added to the respective wells and incubated at RT for 10 min.
4. Wells were washed with wash buffer 3 times with the diluted wash buffer.
5. In all the wells 2 drops of enzyme conjugate was added and incubated for 10 min. at RT.
6. Again the wells were washed with wash buffer 3 times and slapped against paper towel to remove all liquid.
7. In all wells 2 drops of conjugate was added and incubated at RT for 5 min.
8. Two drops of stop solution was added to the wells and mixed by tapping strip holder.
9. Plate was read at 450/620 nm within 1 hour of adding stop solution.

Positive and negative control was also included in the assay.

5.2.4 Indirect hemagglutination assay :

Principle:

An indirect hemagglutination test for the diagnosis of leptospirosis is described; it uses a soluble antigen to sensitize sheep erythrocytes which are then fixed with glutaraldehyde. This is a quantitative assay for the quantification of anti-leptospira antibody. This test can be done in microtitre plates or tubes.

Procedure:

Antigen preparation

Leptospire of serovar E-patoc/pomona were grown in EMJH medium which contained 2–3% rabbit serum. Cultures were kept at room temperature for 5–7 days. The organisms were observed under a dark field microscope. The amount of suitable leptospire was approximately 3+ to 4+ with active movement and no clumping was observed.

Heat extracted antigen (HEA):

After checking for growth under dark ground microscope, the leptospire is killed with formalin (0.5% final conc.).

After 1 hour the killed leptospira culture is heated in a boiling water bath for 30 min with shaking every 5 min. After cooling to room temperature, the culture is centrifuged at 10000 rpm for 10 min. The supernatant is used as antigen.[201]

Profiling of Heat extracted antigen:

Total protein estimation of heat extracted antigen by Biuret assay:

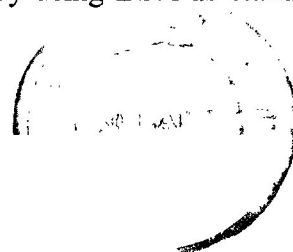
Principle: The Biuret assay is specifically intended for substances with two or more peptide bonds. With it, a violet color complex is being formed between the Cu^{2+} Biuret reagent and the nitrogen atoms involved in the peptide bond.

Materials:

1. Protein standard 5 mg/mL Bovine serum albumin (BSA). (Prepare fresh)
2. Biuret reagent (Dissolve 3 g of copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and 9 g of potassium tartrate in 500 mL of 0.2 mol/L sodium hydroxide; add 5 g potassium iodide and make volume up to 1 L with 0.2 mol/L sodium hydroxide)
3. Diluent: Distilled water.
4. Water bath at 37°C .

The method is as follows;

Different dilutions of Standard BSA were prepared. The BSA range was 0.5 – 2.5 mg/mL. Add 3 mL of biuret reagent to 2 mL of protein solution, mix and incubate at 37°C for 10 min. Cool and read extinction at 540 nm. The Optical density (OD) 540 nm measured with a colorimeter, the concentration of HEA (patoc/pomona) was estimated by comparing the OD values to a Standard Graph plotted by using BSA as standard and Excel software.



Total protein concentration of HEA of serovar pomona and patoc were 1.2 mg/mL and 1.7 mg/mL respectively.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS- PAGE)

Principle: Sodium Dodecyl Sulphate-Polyacrylamide Gel electrophoresis involves the use of sodium dodecyl sulphate (SDS), a strong anionic detergent. It is used in combination with β -mercaptoethanol (reducing agent) to dissociate the proteins. The latter breaks disulphide linkages resulting in primary linear structure of the protein, whereas SDS imparts a net negative charge. Migration of the polypeptides is independent of shape and charge but separation is indeed based on the molecular weight/size. Thus, by using markers of known molecular weights it is possible to estimate molecular weight of polypeptides or proteins.

Materials:

- i Acryl amide
- ii SDS
- iii Tris-HCl
- iv Ammonium per sulfate
- v TEMED
- vi Glycine
- vii Coomassie blue
- viii Methanol
- ix Glacial acetic acid

x Glycerol

xi β -mercaptoethanol

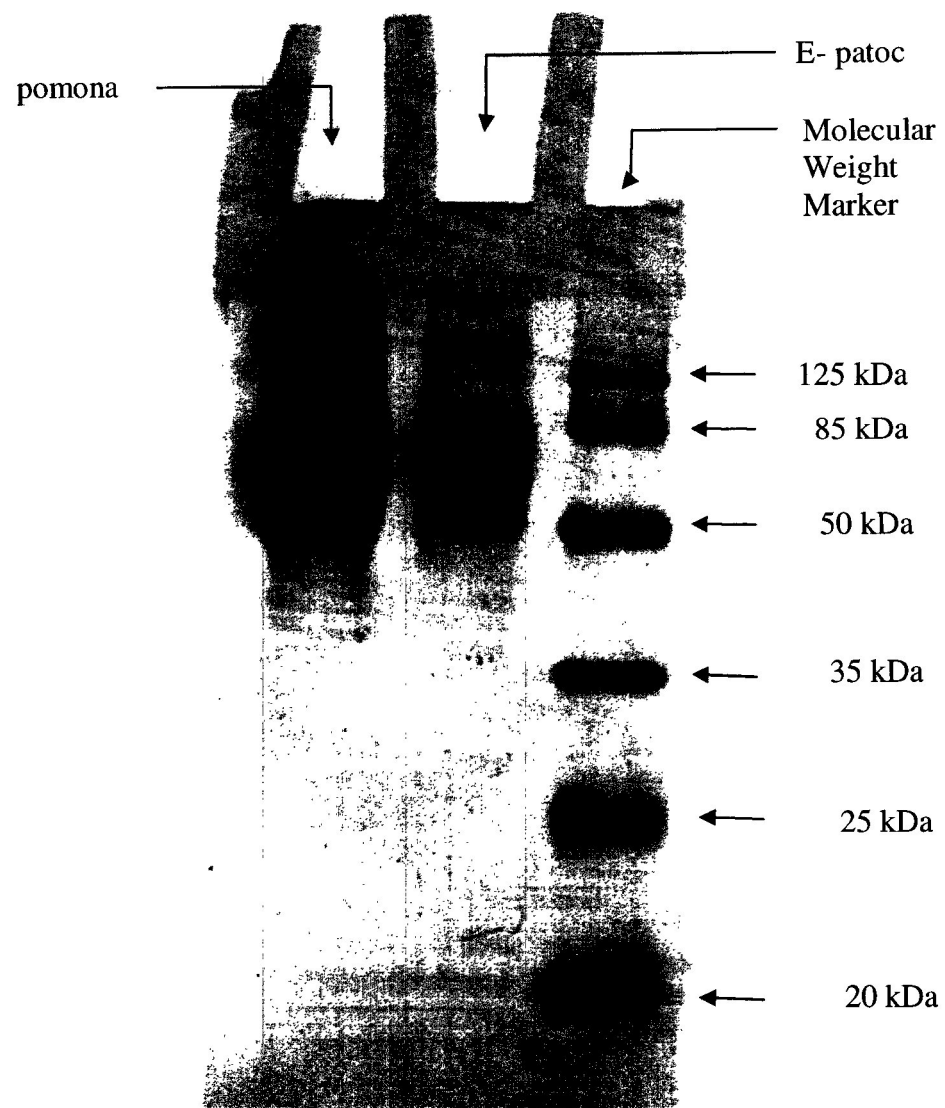
Procedure:

SDS-PAGE was performed using vertical slab gel apparatus and run at 150 V for 2 h. The heat extracted antigen was dissolved in sample lysis buffer containing 0.5 mM EDTA, 0.5% SDS, 0.005% bromophenol blue and 21% Glycerol pH 6.8. Samples as well as protein markers of known molecular weights were then applied to 10% Polyacrylamide gel. Stacking gel of 5% acrylamide was used. After electrophoresis, the protein bands were stained using Coomassie Brilliant Blue (CBB). All the reagents and buffers used in SDS-PAGE are described in Appendix.

Observation:

The bands between 50 and 85 molecular weight of kDa were detected in the antigen preparations from serovar pomona and patoc. (As shown in the Figure 10)

Figure 10: Coomassie brilliant blue (CBB) staining of SDS-PAGE of heat extra antigen revealed broad band between 50 and 85 kDa.



Sensitization of sheep red blood cells (SRBC):

Under all aseptic precautions, blood was collected from sheep in centrifuge tubes containing equal amount of Alsever's solution (Appendix) with the help of veterinary staff.

After collection sheep blood was centrifuged at 1500 rpm for 10 min. Supernatant

down were washed 3 times at 5,000 rpm for 5 minutes at 4°C.

then adjusted to 10% SRBC with Phosphate buffer saline (PBS) pH 7.2 (Appendix). 2.5% glutaraldehyde was added to 1 in 4 of the volume of suspension.

The mixture was rotated for 2 hours at room temperature. After washing 3 times with physiological saline solution, the cell suspension was adjusted to 10% with PBS pH 7.2 containing 0.1% sodium azide. The preserved SRBC can be stored at 4°C for a month. Three hundred μ L of the preserved SRBC were washed with PBS pH 7.2 then an equal volume of 1:20,000 tannic acid was added. The mixture was incubated at 37°C for 30 min.

After washing twice with PBS pH 6.4 (Appendix), the cells were adjusted to 10% suspension. Optimal dilution of each antigen was added. The mixture was incubated at 37°C for 30 minutes, then washed twice with PBS pH 7.2 and adjusted to 0.5% SRBC suspension. PBS pH 7.2 containing 0.5% bovine serum albumin and 0.1% sodium azide. Sensitized RBC's were stored at 4°C for further use.[201]

Assay procedure:

All the sera to be tested were inactivated at 56°C for 30 minutes. Twenty-five μ L of phosphate buffer saline was added to all the wells of V-bottom 96 well microplate. In the first column 25 μ L of test sera was added, and then it was serially diluted two-fold dilution. Final 25 μ L discarded. Twenty-five μ L of sensitized SRBC were added to each well. The mixture was shaken immediately in the plate for 15–30 seconds and put in a moisture box to prevent evaporation. The plate was incubated at 37°C for 1 hour, and the agglutination pattern was then read. Control

serum dilution giving $\geq 2+$ hemagglutination. Positive and negative reference sera included in every batch tested.[201]

5.2.5 Indirect Immunofluorescence Antibody Technique (IFA):

Principle:

The Indirect Immunofluorescence antibody test is a double-layered technique. An unlabelled antibody (sample) is applied directly to the antigen bound to a slide and then treated with a fluorochrome-conjugated anti-immunoglobulin. The test is used to perform to detect specific antibody response within a serum sample.

Antigen preparation

Cultivation of Serovar E-patoc, tarrasovi + pyrogene and pomona was used to prepare antigen. The strain was grown in EMJH medium containing 10% rabbit serum and incubated at room temperature for seven days. On the 7th day, the culture was examined for density, auto-agglutination and contamination by dark field microscopy, plating and routine culture. The culture was centrifuged at 7,000 rpm for 10 minutes. The sediment was washed three times with 0.85% physiological saline (Appendix 1). The suspension was adjusted to a suitable concentration of approximately 10^8 cells/mL.

Assay procedure:

A drop of 10 μ L of a suitable culture was placed on each of 12 circular areas of a microscopic slide, using a micropipette and was allowed to dry at room temperature.

temperature.

The antigen IFA slide was covered with 10 μ L of diluted serum (1:80) and incubated in a moist chamber at 37°C for 30 minutes. The smear was washed three times with PBS pH 7.4 and air-dried. Finally the smear was treated with 10 μ L of diluted (Fluorescein iso-thiocyanate) conjugated antihuman immunoglobulin IgM (Sigma) (1:80) and incubated in a moist chamber at 37°C for 30 minutes. The slide was washed three times with PBS and mounted with glycerol buffer. A thin cover slip (22 $\times$ 22 mm) was placed on the preparation.

Fluorescence microscopy was conducted with a fluorescence microscope under the oil immersion lens (Carl Zeiss). A positive reaction showed fluorescent green complexes.[202]

5.2.6 Real Time PCR:

Principle:

Real-Time Polymerase Chain Reaction is a laboratory technique based on the polymerase chain reaction, which is used to amplify and simultaneously detect a target nucleic acid molecule. It enables both detection and quantification of a specific sequence in a sample.[193]

Extraction of DNA

1. In tube 300 μ L lysis solution was added, in the same tube 100 μ L serum sample was added.
2. The tube was vortexed properly and incubated at 65°C for 5 min.

3. The tube was then centrifuge at 12000g for 1 min. ,
added to the tube.
4. The tube was vortexed properly to insure proper mixing and incubated at temperature for 3 min. This step was repeated 1 more time. The tube centrifuged at 5000g for 1 min.
5. Supernatant was discarded using barrier tips. 300 μ L of washing solution added to the tube. It was vortex vigorously and centrifuge at 8000g for 1 min.
6. Again the supernatant was discarded and 500 μ L of washing solution added. The tube was vortex vigorously and centrifuge at 8000g for 1 min. Supernatant was again discarded.
7. The above step was repeated again to ensure proper washing. And then tube incubated at 65°C for 5 min. with open lid.
8. The pellet was re-suspended in 50 μ L of DNA-eluent to elute DNA present pellet.
9. The tube was incubated at 65°C for 5 min. with intermediate vortexing.
10. The tube was centrifuge at 12000g for 1 min. Supernatant containing DNA collected and transferred to new tube with proper labeling, which was used for amplification.

DNA amplification:

Real Time PCR assay was performed using TaqMan[®] chemistry in a volume of 25 μ L of PCR reaction mixture (Primer Design, UK). The reaction was carried out on a Cycler Real Time PCR Instrument (Cepheid, USA). Primers used in this study were Outer Membrane protein LipL32 Primers.

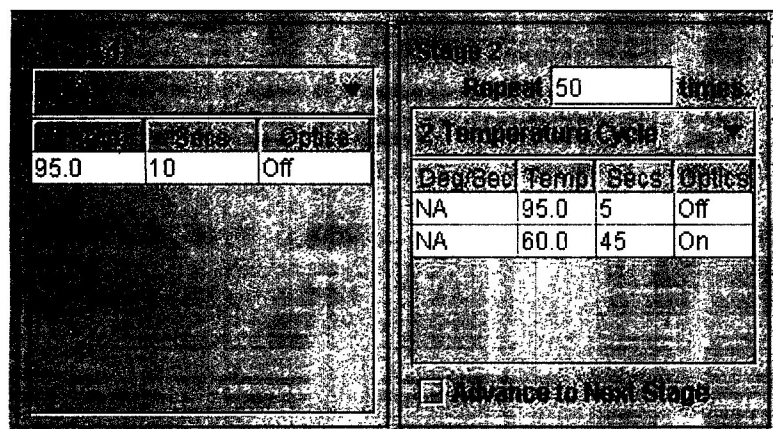
Table 4: PCR reaction mixture for DNA amplification using Real-Time PCR

Reagents	Volume
Premix Ex Taq™ (2 X)	12.5 µL
Primer-probe solution	1 µL
Template	5 µL
PCR grade water	6.5 µL
Total	25 µL

The tube was then loaded on the smart cycler machine. The cycling conditions were as, initial denaturation at 95°C for 10 minutes, followed by 50 cycles of denaturation at 95°C for 10 seconds, annealing and extension at 60°C for 60 seconds.(Figure 11)

Fluorogenic data for the amplified DNA was collected during the reaction through the FAM channel.

Figure 11: Cycling conditions for Real Time PCR



One hundred and nineteen human and 1 hundred animal (rodents and dogs) samples sent to the RMRC reference laboratory, Port Blair, Andaman and Nicobar Islands. These tests require laboratory infrastructure dedicated for *Leptospira* culture, reference serovars and technical expertise.

Principle:

MAT is a standard serology agglutination test, using live *Leptospira* serovars in volumes to dilutions of a serum sample. Results are given as titres of antibodies. This will work with any species of mammalian serum. It is the gold standard *Leptospira* test. MAT measures IgM immunoglobulin rather than IgG as in ELISA and this enables MAT to give a better indication of recent infection. MAT is the definitive reference on serum for pathogenic *Leptospira*s. Cross reactions between sera groups and serovars are common and the highest tittered serovar is taken as the infecting organism.[203]

Assay procedure:

Panel of Antigens: The *Leptospira* antigen battery used for the tests was as follows: *australis*, *bankang*, *ballum*, *canicola*, *grippityphosa*, *hebdomadis*, *icola*, *pomona*, *icterohaemorrhagiae*, *pyrogenes*, *bataviae* and *tarrasovi*.

Preparation of Antigens:

The stock culture collection of *Leptospira*s is best maintained in screw-capped test tubes containing 5–6 mL of liquid medium. Fresh sub cultures are made by inoculating 0.1 mL from the each strain / serovar into tubes. At the same time, a loop-full of the culture should be examined by dark field microscopy to confirm the presence of *Leptospira*s and the absence of contamination and it should be free from “breeding

after 5–7 days. The cultures used as antigens should be checked by MAT & homologous anti-sera frequently for quality control.

Live Antigens:

The culture may be used directly as the antigen. The antigens can be used up to 1 week after 5–7 days of incubation.

Density of the antigens:

Well grown culture, a minimum density of $2-3 \times 10^8$ leptospire per mL should be used as an antigen. The density can be determined by direct counting, using spectrophotometry or by McFarland's scale.

Procedure:

- Fill all 96 wells of microtitre plate with 50 μ L PBS
- Add another 140 μ L PBS to the wells of column 2
- Add 10 μ L of serum to the wells of column 2 (dilution is now 1:20). Mix and discard 100 μ L from last well.
- Dilute by pipetting 50 μ L from 1 well to the next discard the final 50 μ L (2- >3->4- >5- >6- >7 - >8 ->9 ->10 ->11 ->12)
- Add 50 μ L leptospiral culture to all wells
- Mix thoroughly on a micro shaker
- Incubate for 2–4 hours at 30°C

Reading of the test results:

agglutination.

For observation, 1 drop of mixture is transferred with a platinum loop or pipette from well to a microscopic slide and examined under dark field microscope with 20× obj without cover slip.

The titre is the dilution that gives 50% agglutination, leaving 50% of cells free. Control with a control suspension of leptospire diluted 1:2 in PBS without serum

Statistical Analysis:

The entire tests that were carried out for this study were compared with the reference technique using statistical analysis parameters as follows:[204]

Sensitivity (%): $\Sigma TP \times 100 / \Sigma TP + \Sigma FN$

Specificity (%): $\Sigma TN \times 100 / \Sigma TN + \Sigma FP$

Positive predictive value (%): $\Sigma TP \times 100 / \Sigma TP + \Sigma FP$

Negative predictive value (%): $\Sigma TN \times 100 / \Sigma TN + \Sigma FN$

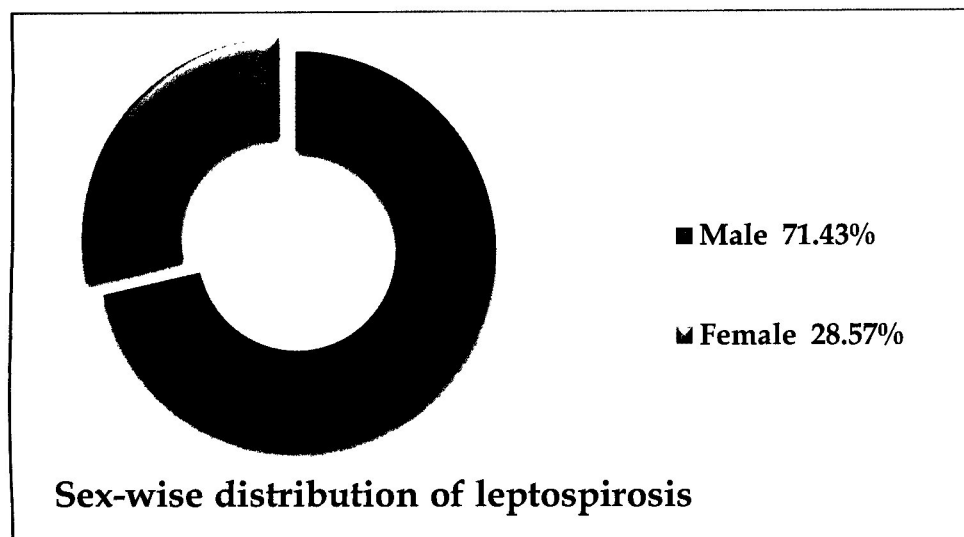
*Observation
&
Results*

6 RESULTS.

A total of 119 human samples and 100 animal (rodent and dog) samples were analysed at the Nodal Laboratory for Re-emerging Diseases, Haffkine Institute, Mumbai to determine the prevalence of leptospirosis in humans and animals using conventional and molecular diagnostic tools.

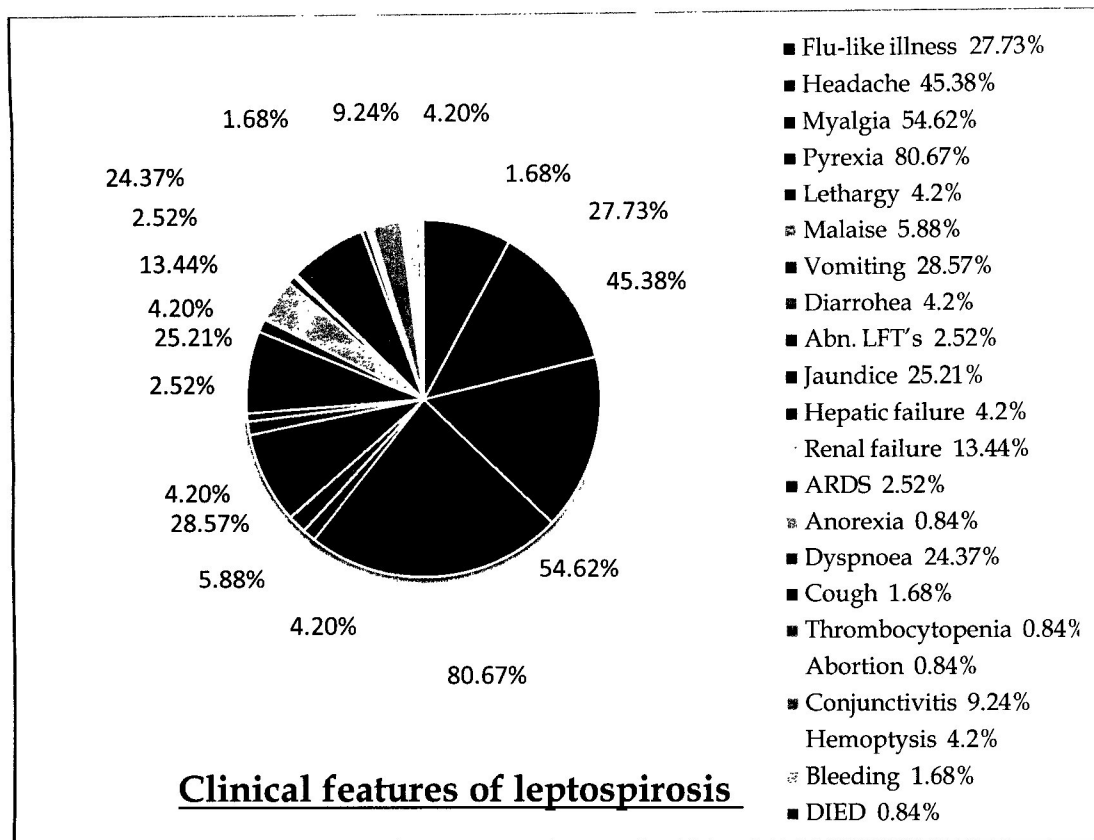
Of the 119 human clinical samples we encountered, 85 (71.43%) of the patients were male, while 34 (28.57%) were females as shown in Figure 12.

Figure 12: Sex-wise distribution of suspected cases of leptospirosis



On sorting the data on the basis of age, the maximum numbers of patients (57%) were from the age group of 25–50. This age group comprises of the occupationally active population.

The patients commonly presented with a triad of symptoms: fever, headache and myalgia. Fever was the most common symptom seen in 81% patients. Figure 13 shows the distribution of clinical features.



The present study was carried out to evaluate risk groups and risk factors in determining the prevalence of leptospirosis. Different risk groups and risk factors for individuals were found during this study are mentioned in Table 5. In this study percent positivity shown by different risk group (occupational) population was 23.53 % (28). Major factors are associated with leptospirosis. In our study, the percent positivity of acute leptospirosis due to water contact was 29.41% (35). Leptospirosis acquired because of contact with animals showed percent positivity of 11.76% (14). Recreational activities also seem to be the cause of leptospirosis in our study with a percent positivity of 8.40% (2). Exposure due to different risk group and risk factors are shown in Figure 14. Out of 119 samples, 28 (23.53%) were found to belong to the risk group for leptospirosis. The highest positivity against leptospirosis was 8.40% (10) seen amongst students,

(6) amongst outdoor workers, 5.04% (6) amongst housewives, 2.52% (3) amongst farmers, 1.68% (2) amongst medical personnel and 0.84% [8] amongst sewage workers. Considering the positivity among the risk group population, the highest positivity for leptospirosis was seen among students 35.71% (10) followed by housewives 21.42%, outdoor workers 21.42% (6), farmers 10.71% (3), medical personnel 7.14% (5) and sewage workers 3.57% [8]. The distribution of different risk groups against leptospirosis positivity is shown in Figure 15.

with Leptospirosis

Characteristics		No. (%)
AGE (YEARS)	<25	38 (31.93 %)
	25–50	68 (57.14 %)
	>50	13 (10.94 %)
GENDER	Male	85 (71.43 %)
	Female	34 (28.57 %)
EXPOSURE TYPE	Occupational	28 (23.53 %)
	Water-related	35 (29.41 %)
	Animal-related	14 (11.76 %)
	Recreational	2 (1.68 %)
SYMPTOMS	Flu-like illness	33 (27.73 %)
	Headache	54 (45.38 %)
	Myalgia	65 (54.62 %)
	Pyrexia	96 (80.67 %)
	Lethargy	5 (4.20 %)
	Malaise	7 (5.88 %)
	Vomiting	34 (28.57 %)
	Diarrohea	5 (4.20 %)
	Abn. LFT's	3 (2.52 %)
	Jaundice	30 (%)
	Hepatic failure	5 (4.20 %)
	Renal failure	16 (%)
	ARDS	3 (2.52 %)
	Anorexia	1 (0.84 %)
	Dyspnoea	29 (%)
	Cough	2 (1.68 %)
	Thrombocytopenia	1 (0.84 %)
	Abortion	1 (0.84 %)
SIGNS	Conjunctivitis	11 (9.24 %)
	Hemoptysis	5 (4.20 %)
	Bleeding	2 (1.68 %)
	DIED	1 (0.84 %)

Occupational: Student-10, Housewife-6, Outdoor-6, Farmers-3, Medical-2, S worker-1.

Water related: Rain water-19, Sewage-4, Pond-2, River-1, Canal-1, Farming-Unknown-7.

Animal related: Unknown-7, Farm live stock-3, Rats-2, Dogs-2.

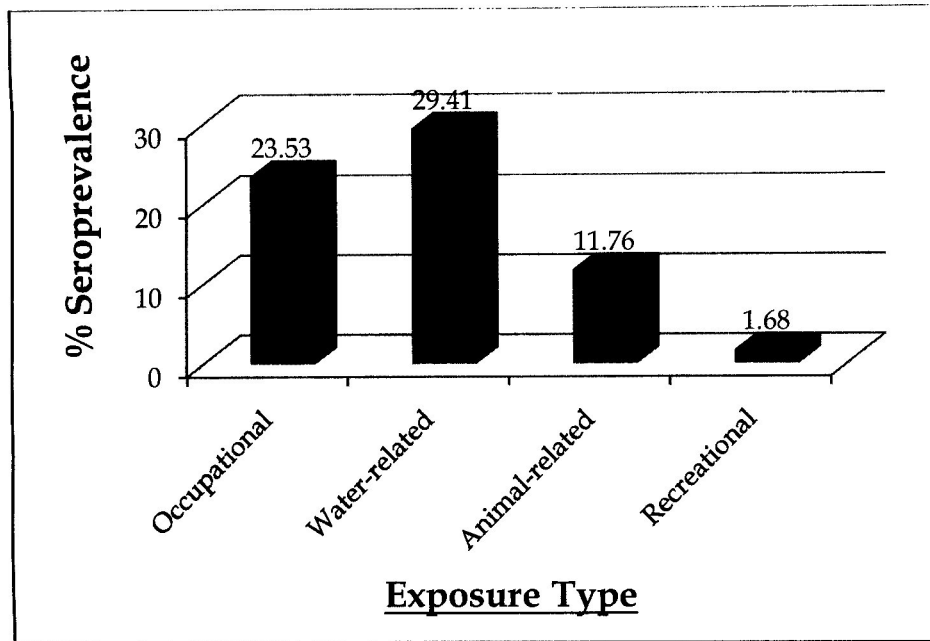
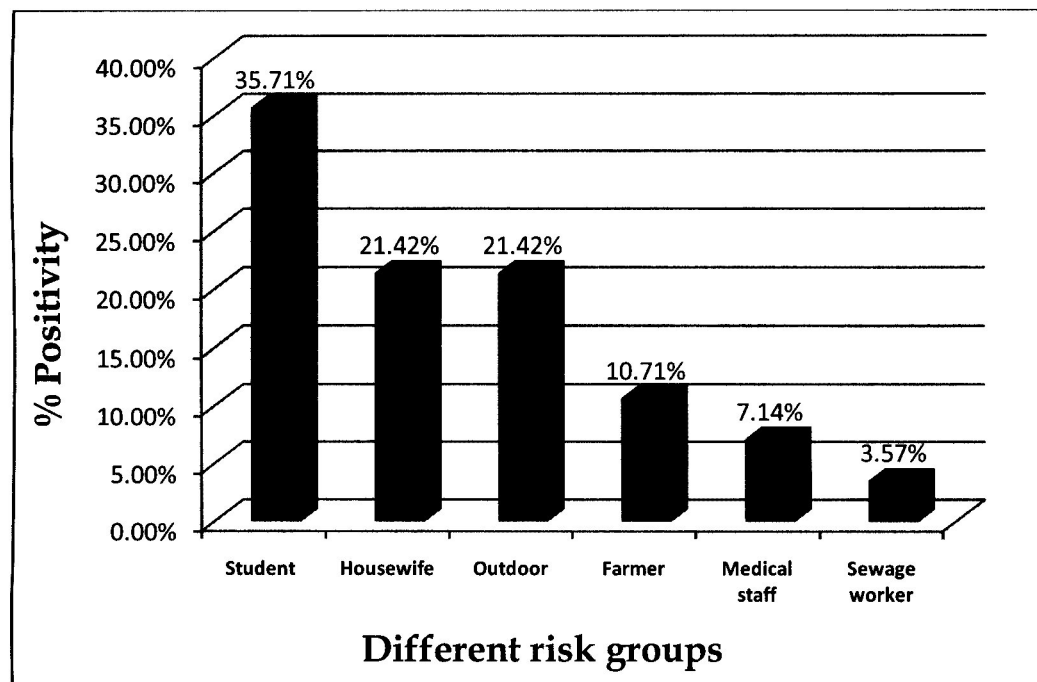


Figure 15: The distribution of different risk groups



Leptospirosis in different risk group was analyzed using different diagnostic tool percent positivity shown by different tools are categorized into Figure 16. Out of :

group samples, 23 (62.14%) were found to be positive with latex agglutination test. (64.29%) samples were positive for IgM antibodies against *leptospira* using IgM I method, 14 (50%) samples were positive with IHA, 14 (50%) samples were positive IFA and 15(53.57%) were positive for leptospiral DNA using Real Time PCR. them were negative by Microscopic agglutination test (MAT).

One of the objectives this study was to find the source of leptospirosis infection in carrier animals (rodents and dogs). A total of 12 urine samples from rodents and 6 samples from dogs were collected and cultured. 12 urine samples from rodents negative for isolation; however, 5 (41.66%) of them were found to be positive with Time PCR performed on DNA extracted from urine sediment. All urine samples dogs were negative by both Real Time PCR and culture techniques. All positive samples from rodents were found to contain DNA from pathogenic *leptospira* confirmed by using Outer Membrane protein LipL32 primers that can only pathogenic *leptospira* using Real Time PCR.

Prevalence of leptospirosis in human as well as animal in and around Mumbai region 1 of the objectives of our study.

1. A) Seroprevalence of leptospirosis in humans was carried out using different diagnostic tools. Out of the referred 1049 samples, Real Time PCR was performed 825 samples, of which 142 (17.21%) were positive for the test. ELISA was performed 224 samples, of which 101 (45.08%) were positive. Out of 119 samples, 99 (83 samples were positive with latex agglutination test. Indirect hemagglutination confirmed 56 (47.05%) samples for presence of anti *leptospira* antibodies. A total

followed by icterohemorrhagiae, bataviae, pomona, tarrassovi and canicola (Figure

Figure 16: Seroprevalence in humans determined using different diagnostic tools

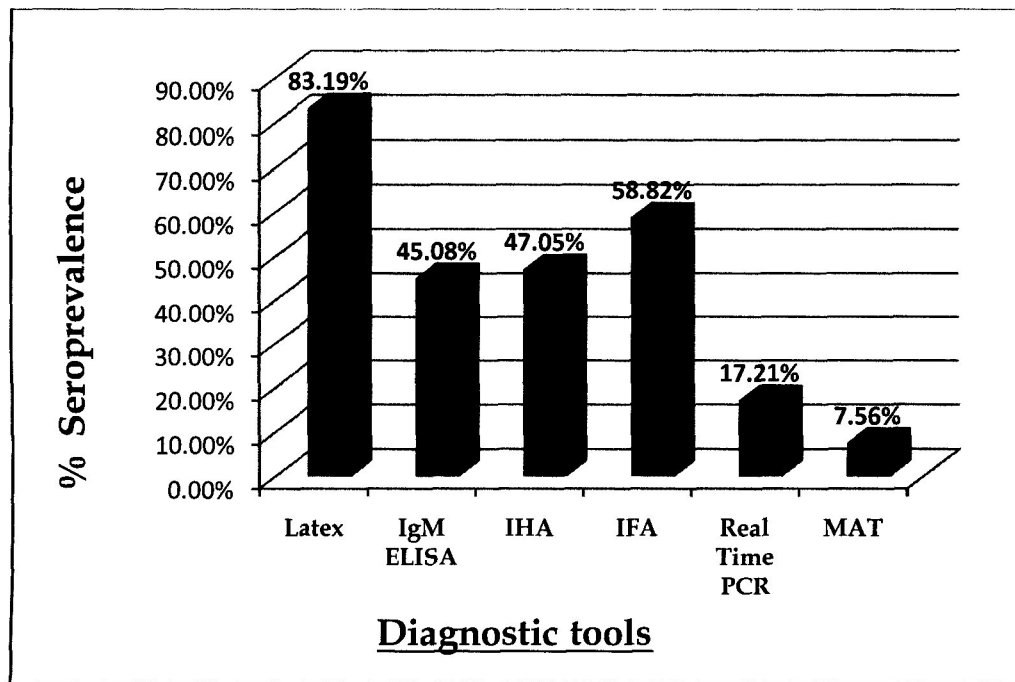
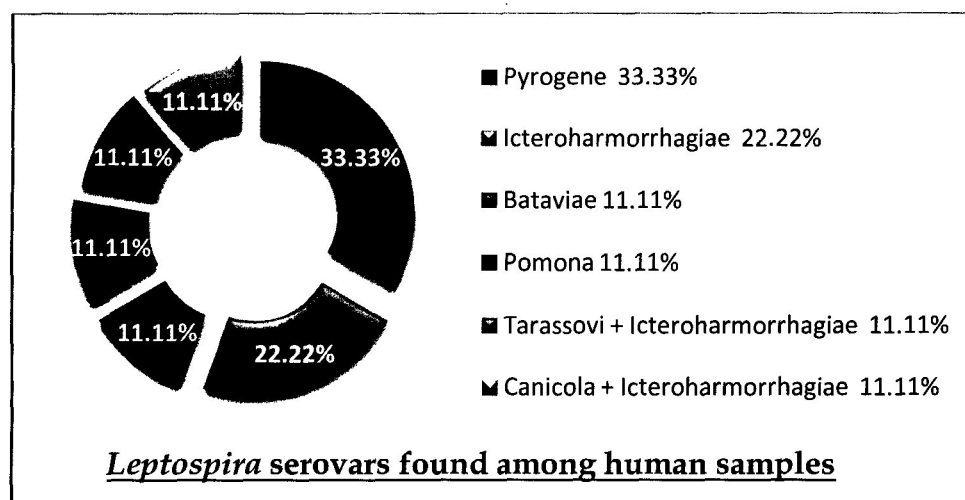
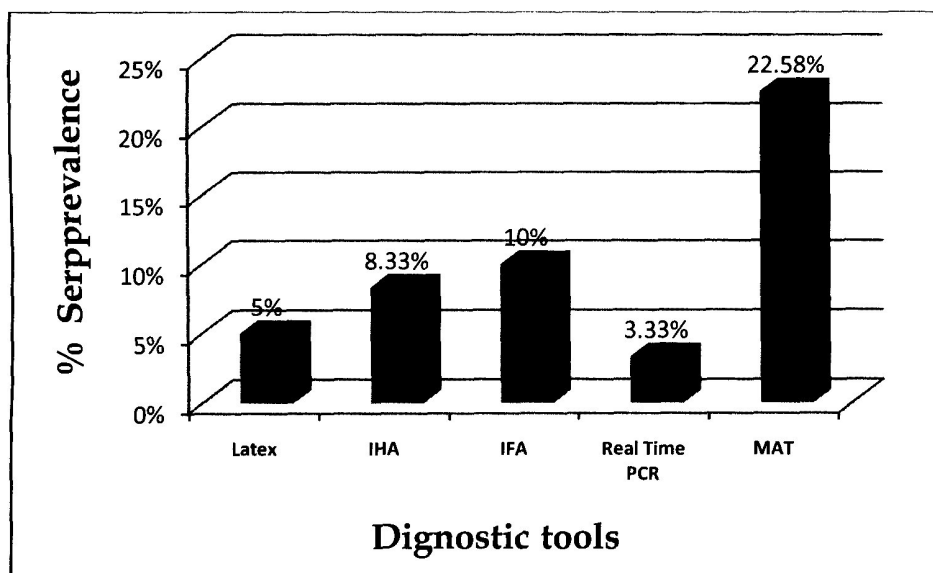


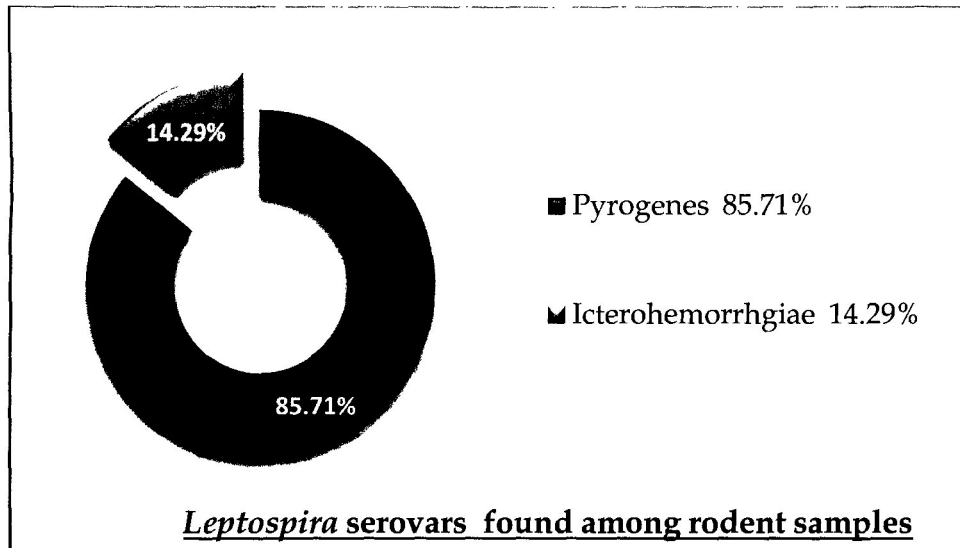
Figure 17: Leptospira serovars found among human samples using MAT



different wards of Brihan Mumbai Municipal Corporation (BMC) with the help of staff. All 60 samples were tested with latex agglutination test and 3 (5%) were positive for the test. IHA showed positivity for 5 (8.33%) samples, while 6 (10%) samples tested positive with IFA. In 2 (3.33%) samples leptospiral DNA was detected using Time PCR assay. Out of 60 samples, only 31 were processed with MAT as rest samples were spilled during transportation while samples were being sent to RMRC Blair, Andaman and Nicobar Islands. Out of 31 samples, 7 (22.58%) samples were positive for the test. Seroprevalence in rodents detected using different tools is shown in Figure 18. The most common serovar detected was pyrogenes, followed by icterohemorrhagiae (Figure 19).

Figure 18: Seroprevalence in rodents detected using different diagnostic tools





1. C) A total of 40 blood samples were collected from dogs suspected of leptospirosis. All samples were tested with different diagnostic tools for diagnosis of leptospirosis. A total of 7 (17.5%) samples showed positivity with latex agglutination test, Anti-leptospira antibodies were detected in 18 (45%) samples with IHA and when tested against IHA, 18 (45%) samples were found to be positive. All of the samples were negative for the presence of *leptospira* DNA. All 40 samples were processed with MAT, of which 8 (20%) were found to be positive for the test (as shown in Figure 20). The most common serovar detected was pyrogenes, followed by icterohemorrhgiae (Mixed infection) as shown in Figure 21.

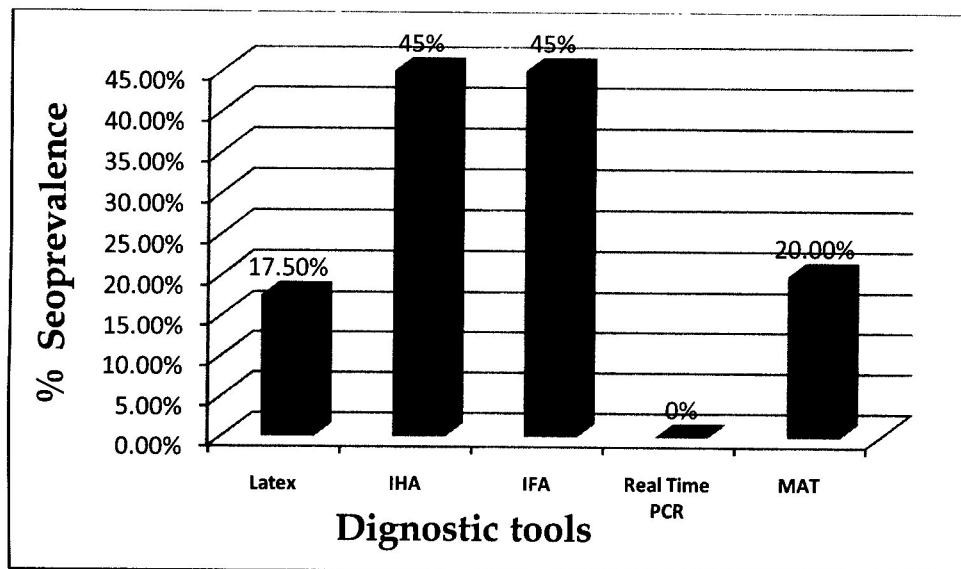
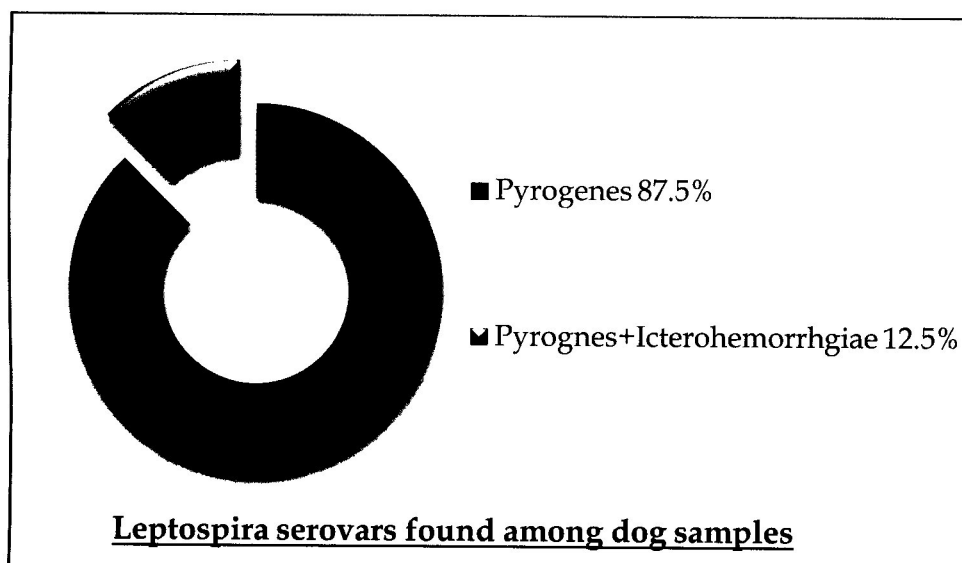


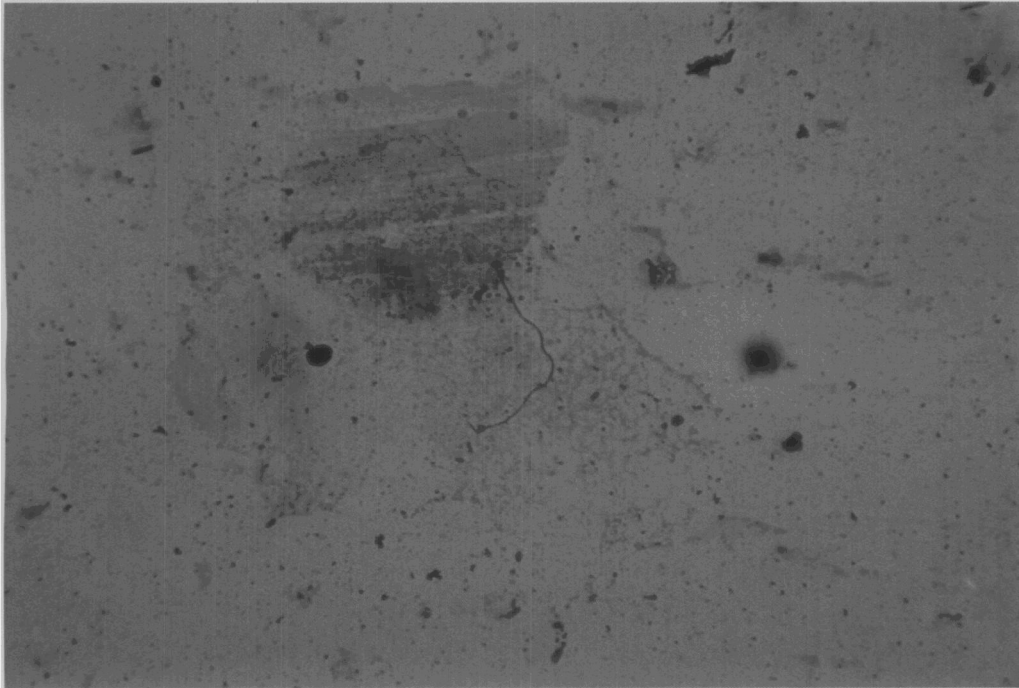
Figure 21: Leptospira serovars found among dog sera using MAT



Leptospira isolation allows definitive diagnosis of infection. One of the objectives study was to isolate the organism from blood and urine samples.

into EMJH medium. Out of them 4 (8%) were positive for isolation. The cultures were confirmed to be leptospires by morphology; Fontana's staining showed black spirochetes against pale yellow or brown colour background as shown in Figure 22.

Figure 22: Culture stained with Fontana's staining



The characterization of the isolate to species level was one of the motives. This was done using extraction of DNA from all 4 samples and its amplification on the Real time PCR. All isolates were differentiated to their species level using Outer membrane protein LipL32 primers, which can detect only pathogenic *leptospira* using Real Time PCR assay. All the isolates were belonging to pathogenic *leptospira*.

All blood samples collected from dogs and rodents were inoculated into EMJH medium. However, no isolation was possible from any of them. Twelve urine samples from

rodents and six urine samples from dogs were diluted and inoculated into EMJH m

All of them found to be negative for isolation.

In this study an attempt was made for comparative analysis of different diagnostic for diagnosis of leptospirosis in humans and animals.

1. A) A total of 119 samples from humans were analyzed using different diagnostic Latex agglutination test were carried out for all of them, positive agglutination observed in 99 samples. To determine performance of latex agglutination test for diagnosis of leptospirosis, it was compared with reference technique, MAT. The results are shown in Table 6.

Table 6: Comparison of Latex Agglutination Test with MAT (Humans)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Latex Agglutination Test	Positive	9	90	99
	Negative	0	20	20
	Total	9	110	119
Index		Value		
Sensitivity		100%		
Specificity		18.18%		
Positive Predictive Value		9.09%		
Negative Predictive Value		100%		

ELISA was also used to detect IgM antibody against *leptospira*. A total of 84 out serum samples have shown presence of IgM antibody against *leptospira*. ELISA were compared with gold standard test MAT to check efficacy of the test. The results shown in Table 7.

Table 7: Comparison of ELISA with MAT (Humans)

		Std. Criteria (MAT)		Total
		Positive	Negative	
ELISA	Positive	7	77	84
	Negative	2	33	35
	Total	9	110	119
Index		Value		
Sensitivity		77.77%		
Specificity		30%		
Positive Predictive Value		8.33%		
Negative Predictive Value		94.28%		

Diagnosis of leptospirosis was also done using Indirect Hemagglutination Assay (II Figure 23). Anti-*leptospira* antibodies were found in 56 out of 119. The distribution of the HA and MAT titres for the 119 sera from patients is shown in Table 8.

Table 8: Distribution of 119 human sera from suspected cases of leptospirosis by MAT and indirect hemagglutination titres.

MAT titres	No. of sera	Hemagglutination Titres						
		Negative	1:8	1:16	1:32	1:64	1:128	1:256
Negative	110	59	9	2	21	11	3	5
1:80	7	2	3	-	1	1	-	-
1:160	2	2	-	-	-	-	-	-
Total	119	63	12	2	22	12	3	5

Figure 23: Photograph of IHA carried out on human sera

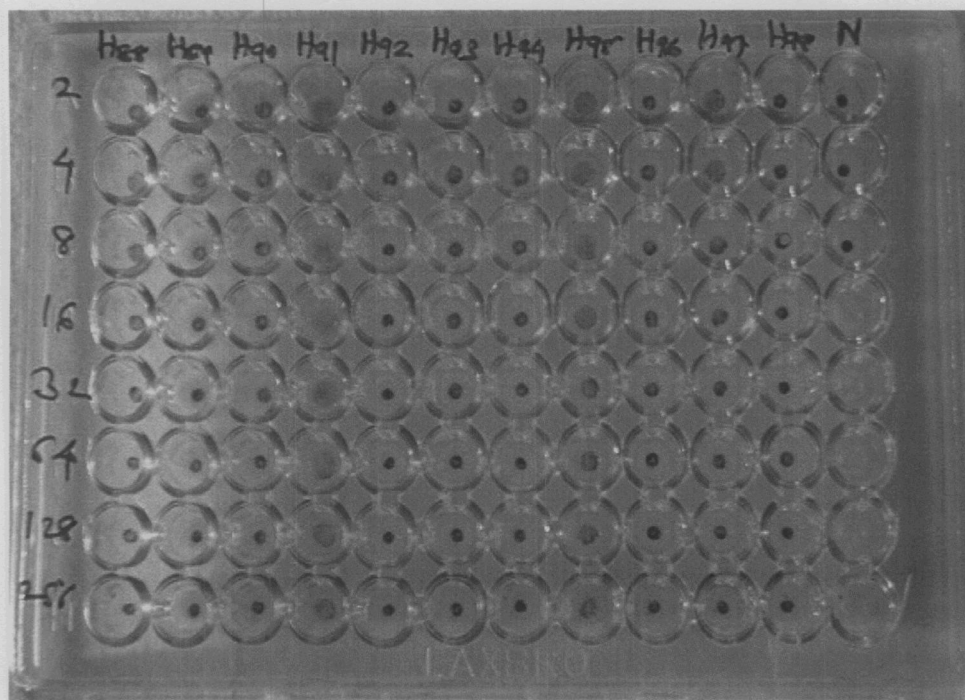
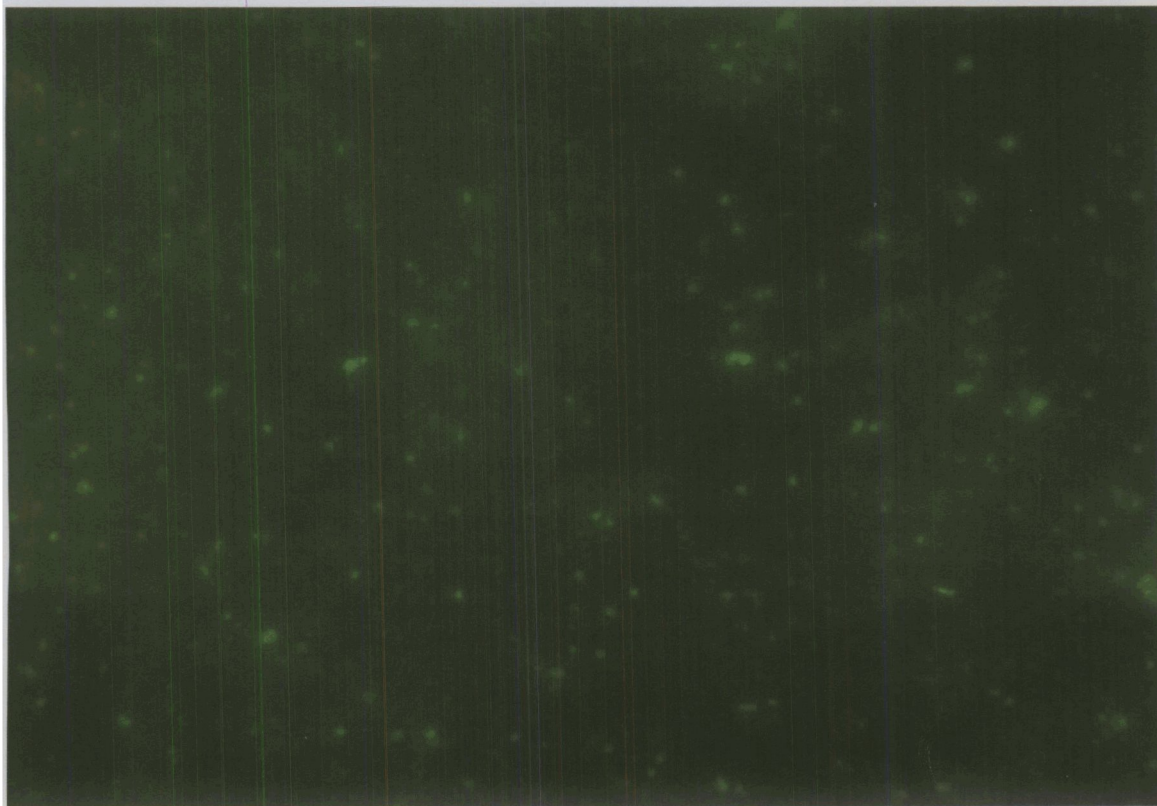


Table 9: Comparison of Indirect Hemagglutination Assay with MAT (Humans)

		Std. Criteria (MAT)		Total
Indirect Hemagglutination Assay		Positive	Negative	
	Positive	5	51	56
	Negative	4	59	63
	Total	9	110	119
Index		Value		
Sensitivity		55.55%		
Specificity		53.63%		
Positive Predictive Value		8.92%		
Negative Predictive Value		93.65%		

Indirect Immunofluorescence Antibody test (IFA) was also carried out to detect the presence of antibodies against *leptospira*. Positive Immunofluorescence as shown in Figure 24 was found in 70 out of 119 samples. The test results were compared with the standard test MAT. The results are shown in Table 10.

fluorescent green particle



**Table 10: Comparison of Indirect Immunofluorescence Antibody Test with MAT
(Humans)**

		Std. Criteria (MAT)		Total
		Positive	Negative	
Indirect Immunofluorescence Antibody Test	Positive	5	65	70
	Negative	4	45	49
	Total	9	110	119
Index		Value		
Sensitivity		55.55%		
Specificity		40.9%		
Positive Predictive Value		7.14%		
Negative Predictive Value		91.83%		

The presence of leptospiral DNA was also detected from 119 serum samples using Real Time PCR technique. 56 samples were found to be positive for the presence of leptospiral DNA. To determine the efficacy of the test, it was compared with MAT. The results are shown in Table 11.

Table 11: Comparison of Real Time PCR with MAT (Humans)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Real Time PCR	Positive	2	54	56
	Negative	7	56	63
	Total	9	110	119
Index				Value
Sensitivity				22.22%
Specificity				50.9%
Positive Predictive Value				3.57%
Negative Predictive Value				88.88%

comparison between two tests was made using 31 sample numbers.

Latex agglutination test was used to detect the anti-*leptospira* antibodies. Only 3 samples were found to be positive for the antibodies. To assess the efficacy of the test, it was compared with reference technique MAT. The results are shown in Table 12.

Table 12: Comparison of Latex agglutination test with MAT (Rodents)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Latex Agglutination Test	Positive	3	0	3
	Negative	4	24	28
	Total	7	24	31
Index		Value		
Sensitivity		42.85%		
Specificity		100%		
Positive Predictive Value		100%		
Negative Predictive Value		85.71%		

IHA was also used for the diagnosis of leptospirosis. Five out of Thirty-one sample found to be positive (as shown in Figure 25) for the test. The distribution of the HA MA titres for the 31 sera from rodents is shown in Table 13.

by MAT and indirect hemagglutination titres

MAT titre	No. of sera	Hemagglutination Titre						
		Negative	1:8	1:16	1:32	1:64	1:128	1:256
Negative	24	24	-	-	-	-	-	-
1:80	1	1	-	-	-	-	-	-
1:160	2	-	1	-	1	-	-	-
1:320	1	1	-	-	-	-	-	-
1:640	3	-	2	1	-	-	-	-
Total	31	26	3	1	1	-	-	-

Figure 25: Photograph of IHA carried out on rodent sera



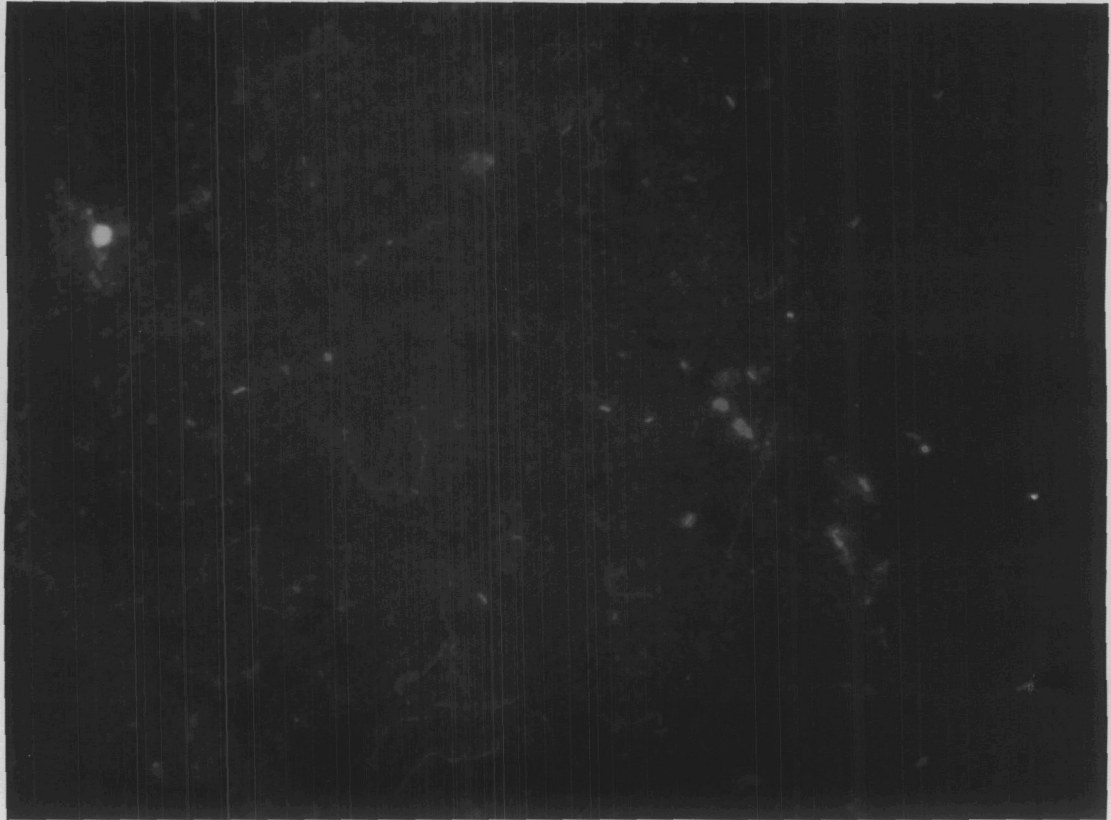
The performance of the indirect hemagglutination assay was evaluated using standard test MAT. The results are shown in Table 14.

Table 14: Comparison of Indirect hemagglutination assay with MAT (Rodents

		Std. Criteria (MAT)		Total
		Positive	Negative	
Indirect Hemagglutination Assay	Positive	5	0	5
	Negative	2	24	26
	Total	7	24	31
Index		Value		
Sensitivity		71.42%		
Specificity		100%		
Positive Predictive Value		100%		
Negative Predictive Value		92.3%		

shown in figure 26. The efficacy of the test was determined by comparing it with standard technique MAT (Table 15).

Figure 26: Photograph of positive rodent sample, leptospire is visualized as fluorescent green particle



**Table 15: Comparison of Indirect Immunofluorescence antibody test with MAT
(Rodents)**

		Std. Criteria (MAT)		Total
		Positive	Negative	
Indirect Immunofluorescence Antibody Test	Positive	6	0	6
	Negative	1	24	25
	Total	7	24	31
	Index	Value		
Sensitivity				85.71%
Specificity				100%
Positive Predictive Value				100%
Negative Predictive Value				96%

The presence of leptospiral DNA from serum samples were detected using Real Time PCR technique. Only 1 out of 31 samples were found to be positive for the presence of Leptospiral DNA. The performance of the test was determined by comparing it with standard test MAT. The results are shown in Table 16.

Table 16: Comparison of Real Time PCR with MAT (Rodents)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Real Time PCR	Positive	0	1	1
	Negative	7	23	30
	Total	7	24	31
Index		Value		
Sensitivity		0%		
Specificity		95.83%		
Positive Predictive Value		0%		
Negative Predictive Value		76.66%		

using conventional and modern diagnostic tools.

Latex agglutination test was carried out on all samples, 7 of them were found positive for the test. The performance of the test was evaluated by comparing it with reference test MAT. The results are shown in Table 17.

Table 17: Comparison of Latex agglutination test with MAT (Dogs)

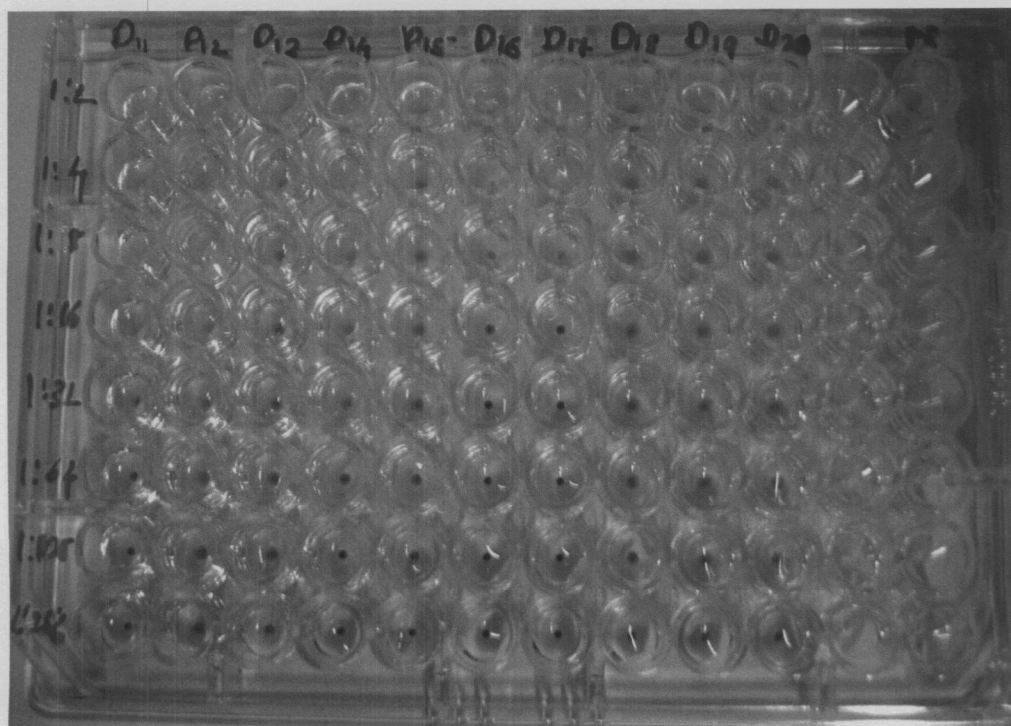
		Std. Criteria (MAT)		Total
		Positive	Negative	
Latex Agglutination Test	Positive	3	4	7
	Negative	5	28	33
	Total	8	32	40
Index		Value		
Sensitivity		37.5%		
Specificity		85.7%		
Positive Predictive Value		42.85%		
Negative Predictive Value		84.84%		

distribution of the HA and MA titres for the 40 sera from dogs is shown in Table 18.

Table 18: Distribution of 40 rodent serum specimens for prevalence of leptospira by MAT and indirect hemagglutination titres

MAT titre	No. of sera	Hemagglutination Titre						
		Negative	1:8	1:16	1:32	1:64	1:128	1:256
Negative	32	22	6	1	2	1	-	
1:40	1	-	1	-	-	-	-	
1:80	3	-	1	1	1	-	-	
1:160	2	-	2	-	-	1	-	
1:320	-	-	-	-	-	-	-	
1:640	2	-	2	-	-	-	-	
Total	40	22	12	2	3	1	-	

Figure 27: Photograph of IHA carried out on dog sera



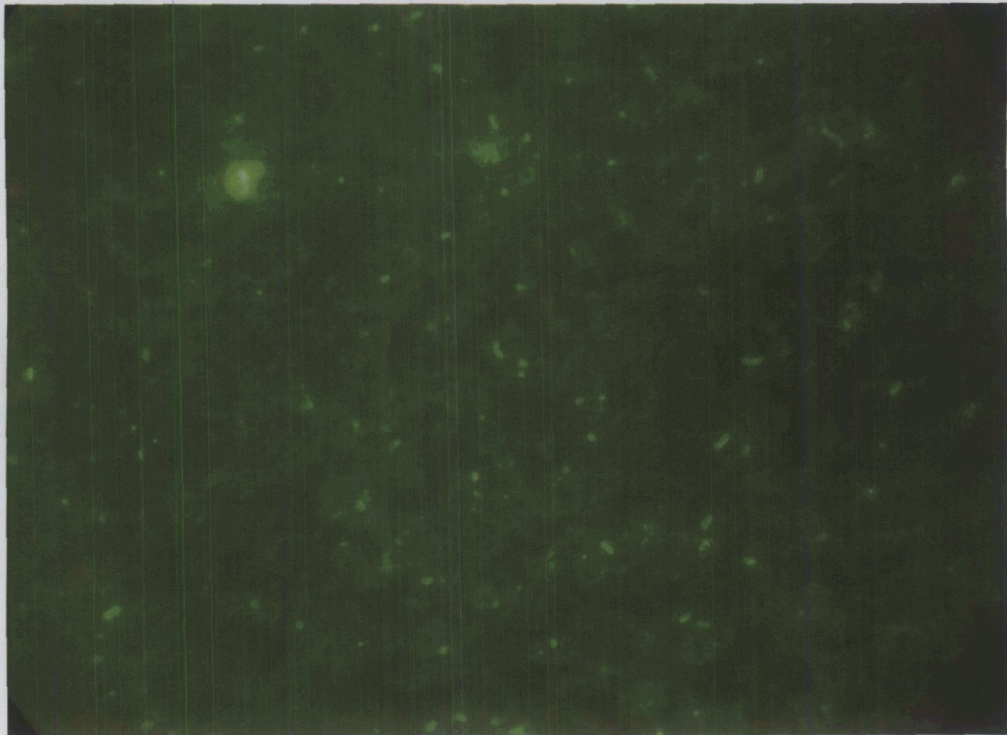
The results of indirect hemagglutination assay were compared with reference test M to determine efficacy of the test. The results are shown in table 19.

Table 19: Comparison of Indirect hemagglutination test with MAT (Dogs)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Indirect Hemagglutination Assay	Positive	8	10	18
	Negative	0	22	22
	Total	8	32	40
Index		Value		
Sensitivity		100%		
Specificity		68.75%		
Positive Predictive Value		44.44%		
Negative Predictive Value		100%		

Out of 40 samples, 18 were found to be positive as shown in Figure 28 with IFA as diagnostic tool. The efficacy of the test was determined by comparing it with standard technique MAT (Table 20).

Figure 28: Photograph of positive rodent sample, leptospire is visualized as fluorescent green particle



(Dogs)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Indirect Immunofluorescence Antibody Test	Positive	7	11	18
	Negative	1	21	22
	Total	8	32	40
Index		Value		
Sensitivity				87.50%
Specificity				100%
Positive Predictive Value				100%
Negative Predictive Value				95.45%

All of the 40 serum samples were found to be negative for leptospiral DNA using Real Time PCR technique. The efficacy of the test was determined using reference technique MAT. The results are shown in Table 21.

Table 21: Comparison of Real Time PCR with MAT (Dogs)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Real Time PCR	Positive	0	0	0
	Negative	8	32	40
	Total	8	32	40
Index		Value		
Sensitivity		0%		
Specificity		100%		
Positive Predictive Value		0%		
Negative Predictive Value		80%		

Discussion

Leptospirosis is now identified as one of the globally emerging infectious diseases, having an increased incidence in tropical locations and in those exposed by virtue of their occupation.

The clinical manifestations are variable, ranging from subclinical infection to hepatorenal failure, severe pulmonary haemorrhage, and death. Humans are incidental hosts of leptospirosis with infection caused by direct and indirect exposure through water and recreational activities. Humans are not considered carriers of the disease. The International Leptospirosis Society estimates that 300000–500000 cases of leptospirosis occur annually around the world with mortality ranging from 5–20%. For leptospirosis, risk groups are certain groups of humans in a population that are likely to be exposed as a result of their occupational/recreational activities. New risk groups may be formed as a result of changes in agricultural or social practices, changes in reservoir animal populations in an area. Occupational exposure is thought to be responsible for 30–50% of cases. Occupations with a high risk of infection include fishermen, workers, coal miners, plumbers, farm workers, veterinarians, pet shop owners, animal workers, meat handlers, slaughterhouse workers, workers in the fishing industry, and the military. From 8–29% of those who work with livestock have antibodies to Leptospira. Recreational activities that increase the risk of leptospirosis include gardening and sports such as canoeing, swimming and white-water rafting. Residents of some rural areas are exposed via rat urine.

Most cases of leptospirosis are asymptomatic or mild. The overall case fatality rate is 5%. The mortality rate varies with the form, and is higher in the elderly. The anicteric form is rarely fatal. The icteric form, which occurs in 5–10% of all patients, has an overall mortality rate of 5–15% and a 54% case fatality rate in severe cases.

uveitis eventually recover kidney or liver function, and full vision.

In addition, there are many factors such as rainwater contact, animal contact and recreational activities responsible for the risk of acquiring leptospirosis. Risk factors can be classified under three broad categories i.e. environmental (type of house, house surroundings, proximity to water bodies, ownership of house and agricultural land), contact with animals (rearing cattle, swine, cat or dogs and rat infestation of houses) and behavioural/occupational factors (participation in agricultural activities, fishing, contact with garbage and sewage, cleaning animals, direct contact with animal urine, and recreational activities such as swimming).

The specific risk group and factors for acquiring leptospiral infection could be unique to each community. An understanding of the transmission cycle of the disease in the community and the modifiable risk behaviors is essential for planning strategies for prevention and control.

Hence, in the present study an attempt was made to determine the risk groups and risk factors for leptospirosis in and around Mumbai region. Out of 119 samples, 28 (23.53%) were found to belong to the risk group for leptospirosis. The highest positivity against leptospirosis was 8.40% (10) seen amongst students, 5.04% (6) amongst outdoor workers, 5.04% (6) amongst housewives, 2.52% (3) amongst farmers, 1.68% (2) amongst medical personnel and 0.84% [8] amongst sewage workers. In our study, it was observed that from the risk group, students seem to be at the highest risk of acquiring leptospirosis followed by housewives and outdoor workers. The higher prevalence in young population (Student) is a universal phenomenon and is generally attributed to their more frequent outdoor activities.[205]

Leptospirosis surveillance carried out by Leptospirosis Reference Laboratory in Queensland and Australia shown similar positivity amongst farmers (2.7%) and lower positivity amongst students (4.5%) as compared to our study. [131] One of the reasons of lower positivity in farmers in present study may be that the study population was primarily urban (study area). One of the studies from Kolenchery, Kerala, India, author reported that the disease was often transmitted in association with activities of daily living. Housewives constituted more than 25% of all cases, which was explained by the practice of washing linens in canals and ponds, and care of domestic animals.[206]

We also made an attempt to determine the risk factors responsible for the leptospirosis. From this study, we observed that rain water contact was the most common risk factor showing percent positivity of 29.41% (35), followed by animal contact 11.76% (14) and recreational activities 1.68 (2). In one of the studies from Japan, the authors have reported leptospirosis cases during recreational activity.[51]

Incidence of leptospirosis is greatly influenced by the rodent population in a geographical region. Once acquired, the organisms colonize the kidney cortex of the rodent for more or less its lifetime without showing a disease syndrome. Rodents are prolific shedders of leptospirae voiding them in urine continuously. Social life styles, particularly the rural life is very conducive for leptospirosis. In most instances all the housing of a villager is a single enclosure, portion of which is shared between him and his livestock. The houses are invariably rodent infested. Therefore, leptospirosis is easily transmitted between human and animal hosts. Bathing, drinking and wading through such water bodies, engaging in water sports, drinking water supply from underground water storage tanks with prevailing rodent borrow is wrought with danger of Leptospirosis. Rodent urine contamination of stagnant water in wet land is another predisposing factor for

Leptospirosis is most common with farmers in India. A spurt in incidence of leptospirosis in human is an annual feature in some developing countries during monsoon. During rainy season rodent urine contaminated drainage material, garbage heaps and manure pits are spilled on the roads which is a public health hazard particularly for those walking bare foot and children who are easily attracted to play in puddles of rain water. Grocery shops, provision stores, hotels and restaurants, roadside dhabas, animal feed mixing plants and flourmills are potential sources of infection. It is a common knowledge in developing countries that food grains and other edible stuff in the grocery stores and the left-over cooked foodstuff in hotels are left open after late hour night duties by the staff. The scenario is well set for rodents to thrive and breed aiding spread of leptospirosis. An in-depth analysis of these predisposing factors as contributory to episodes of leptospirosis is rarely done but the situation is rampant and glaringly threatening.

Dogs as companion animals have a special social status in the urban and rural society. Irrespective whether it is the well looked after and groomed companion animal of the urban elite or the uncared and neglected community street dog, the risk of leptospira infection from dogs to human and other animals is enormous. A small section of the owned dogs do get protection of vaccination, but it is too inadequate.

Leptospirosis is a major zoonosis of the veterinary profession. Handling of sick animals and carrier animals makes the situation more difficult for the veterinary clinician in taking precautions. Those handling animal labour, dystocia's and cases of retention of placenta are more vulnerable. Likewise, butchers, animal handlers and those working in carcass processing units are particularly at considerable risk of leptospirosis.

Consequently to study the transmission dynamics of leptospirosis an attempt was done to

PCR performed on DNA extracted from urine sediment. Although Real time PCR on urine sample was positive, isolation could not be possible because leptospires can be inactivated just after the sample collection and Real time PCR can detect the organism even if they are inactivated.[207]

All urine samples from dogs were negative for both Real Time PCR and isolation techniques. Failure to demonstrate leptospires in the urine of dogs does not necessarily eliminate the possibility that the animal is a chronic renal carrier; it merely indicates that the animal was not excreting detectable numbers of leptospires at the time of testing.[208] In each case, the best sample to test is urine collected before the dog receives the first dose of antibiotics. The present study incorporated samples from dogs, which being already treated at Bombay veterinary college. After antibiotic *therapy* begins, the organism is usually difficult or impossible to find in urine samples. Therefore, to get a diagnostic sample, you have to consider a diagnosis of leptospirosis as soon as the dog presents signs. If leptospires are detected in the urine, the result is definitive; a negative test result means only that leptospires were not detected, even though they might be harbouring them. As reservoir hosts, dogs can shed this organism for months to years after infection, making canicola infection a serious potential problem.

The dog does not serve as an important host for the other serovars that infect dogs e.g. bratislava, pomona and grippotyphosa. In general, infected dogs shed these organisms in their urine for a relatively short time: days to a couple of weeks. Some dogs shed serovar icterohaemorrhagiae for a few months. It's clear that appropriate antibiotic therapy will decrease or eliminate the shedding of live, infectious leptospires in urine. Therefore, it's quite unlikely that dogs will shed leptospires in their urine after a course of appropriate therapy. However, dogs infected with serovar canicola may not be cured by a routine

course of antibiotics; a small percentage of them may shed after the antibiotics are withdrawn.[209] One of the reasons of negative isolation is short time between obtaining and processing samples, as it is important for contaminating microorganism and mainly for leptospira viability. Thiermann and Faine reported that the acidic pH in the urine hinders isolation whereas it inactivates and lyses the leptospira cells less than three hours.[210]

All positive rodent urine samples were found to belong to Pathogenic species of *leptospira*. Characterization was done using LipL32 primers using Real Time PCR.[211]

Hence, it may be inferred that rodents play a major role as vectors for leptospirosis.

The rate of isolation of organism from human blood samples was 8% (4/50). The rate of isolation of leptospires from the culture has been described to be very low due to various reasons including prolonged incubation or frequent media contamination. Prior antibiotic usage might have affected the isolation of organisms in our study as urban population was involved to a larger extent. A similar study by S Chandrasekaran et al. showed similar positivity (8.2%) of culture from Madurai, India.[212]

However, no isolation was possible from any animal blood or urine samples. Justification for negative isolation from animal could be that, the pathogenic leptospires live in the kidney of host animals and are excreted in the urine over a few days or for a long time or lifelong. They may be irregularly shed in the urine as opposed to being continuously shed. Different serovars of leptospires are often associated with, or adapted to, different animal hosts. Adapted species may cause no or only minor clinical problems for the host. However, when the leptospires find their way into another animal species, they may

range in clinical effects from benign to acute. Hence, leptospires that are harmful to humans may be benign to rodents. Other wild and domestic animals can contract and excrete leptospires and will suffer the same range of disease ranging from benign to acute depending on the species of leptospira infecting them. Some species of leptospira are known to be more harmful than others, and many of these harmful species are known to be adapted to certain animal hosts.[213] The percentage of successful isolations may, however, not reflect the true percentage of carriership because a number of cultures were lost due to contamination. Indeed contamination with less fastidious and faster growing microorganisms forms a major limitation of *Leptospira* isolation in spite of the fact that contamination can be kept at minimum by deploying selective growth inhibitors such as 5-Fluorouracil in the culture medium.[214]

Isolates from human samples were characterized to species level using LipL32 primers by Real Time PCR assay. It was observed that all 4 isolates from humans belong to pathogenic species. This is highly sensitive and specific Real-time PCR assay for the detection of pathogenic leptospires. The 423 bp target was amplified from pathogenic strains of *Leptospira* spp., but not from non-pathogenic species of leptospira, and wide range of other clinically significant bacteria and yeasts. The LipL32 primers can also be used in a conventional PCR assay. The analytical sensitivity of the assay was 3 genome copies per reaction in blood and approximately 10 genome equivalents per reaction in urine, comparable to a real-time assay which uses a 16S rRNA gene target.[193]

Leptospirosis is most common in tropical countries such as India, Nicaragua and Thailand. This is mainly due to longer survival of leptospires in environments with warm and humid conditions. However, most tropical countries are also developing countries,

the rainy season. Water-borne transmission has been documented and outbreaks of leptospirosis usually occur after flooding. An outbreak of water-borne leptospirosis in and around Mumbai region occurred followed by record monsoon rainfall on 26th July 2005. Leptospirosis claimed most of the lives along with gastroenteritis, and nearly 2500 people were being treated in hospitals in the Mumbai area. As sero-epidemiological data are not available from Mumbai region; we investigated the prevalence of human leptospirosis in this area.

Therefore this study was designed to determine the seroprevalence of Leptospirosis in humans. Different conventional and modern diagnostic tools were used for the study. A total of 99 (83.19%) samples were positive with latex agglutination test, 68 (54.4%) samples were found to be positive with indirect hemagglutination assay, 70 (58.82%) samples were found positive using Indirect Immunofluorescence antibody test and all samples were tested using Microscopic agglutination test (MAT), of which 9 (7.56%) were found to be positive by taking 1:80 as the significant titre. The most common serovar found was pyrogenes followed by icterohemorrhagiae, bataviae, pomona, tarrassovi and canicola. A similar study conducted in Mumbai post deluge outbreak during 2005 shown presence of similar circulating serovars as per our findings.[129]

Leptospire are maintained in nature by numerous sub-clinically infected wild and domestic animal reservoir hosts that serve as a potential source of infection and illness for humans and other incidental animal hosts. The age-long persistence of *Leptospira* genus is enabled by animal life. Rats are the most common source of infection for humans and act as a reservoir host. Rural farm workers are at high risk; man gets infection accidentally through direct or indirect contact with the infected animal and considered to be a “dead end host”.

dogs. The potential for infection of humans was recognized. Both the canicola and the icterohaemorrhagiae forms were described. Due to vaccination, the prevalence of disease caused by canicola and icterohaemorrhagiae has decreased, while there is increasing evidence that the other serovars are becoming more prevalent in producing disease.

Hence, seroprevalence in animals (rodent and dog) was carried out to determine presence of leptospirosis as an indicator of source of infection.

Prevalence of Leptospirosis in rodents showed 3 (5%) samples positive by latex agglutination test, 5 (8.33%) samples were found to be positive with IHA, 6 (10%) samples were tested positive with Indirect Immunofluorescence antibody test and 2 (3.33%) samples were confirmed for the presence of leptospiral DNA with Real Time PCR. The MAT assay was performed on 31 samples out of 60 samples. Of those, 7 (22.58%) samples were positive for the test (as shown in Fig.4). The most prevalent serovar was pyrogenes (85.71%), followed by icterohemorrhagiae (14.29%). Extensive search on the seroprevalence of leptospirosis in rodent could not find any reference from Mumbai region.

Prevalence of Leptospirosis in dogs showed 7 (17.5%) samples prevalent with latex agglutination test. Positive reaction was seen in, 18 (45%) samples with IHA, 18 (45%) showed positive immunofluorescence with Indirect Immunofluorescence antibody test. No sample was found positive for leptospiral DNA. A dog with negative whole blood PCR results for *Leptospira* is likely not infected if the samples were collected prior to antibiotic therapy. However, whole blood PCR results are only positive early in infection therefore, if leptospirosis is still suspected, MAT testing is recommended.[215] All 40 samples were processed with Microscopic agglutination test (MAT), of which 8 (20%)

with pyrogenes). V. Vijayanand et. al. also reported pyrogenes as one of the few serovars found in dogs in peripheral areas around Chennai, India.[216] Similar observation made in one of the studies on seroprevalence of leptospirosis in animals from Tamil Nadu, India.[217]

Laboratory tests are necessary to confirm the diagnosis of clinically suspected leptospirosis due to its varied symptomatology. Moreover leptospirosis must always be considered during the differential diagnosis of other tropical febrile illness such as malaria, enteric fever, viral hepatitis and dengue which have similar presentation. A confusing array of laboratory tests is described for detection of this spirochetes and antibodies. The conventional tests include direct microscopy, culture and most widely used reference standard method- the microscopic agglutination test. In addition a variety of newer serological tests and those based on molecular techniques have been described.[159] Laboratory diagnosis of leptospirosis is an area poorly understood by many of the workers involved in detection of leptospirosis and surveillance. Selection of the right specimens and tests and the correct interpretation of test results are important in order to provide better treatment at the earliest opportunity. Conventional bacteriological techniques such as isolation of the organisms from clinical specimens or serology using MAT are laborious, time-consuming and require well-established laboratory facilities and skilled manpower. Most institutes or hospitals in developing countries may not have such facilities for serodiagnosis of leptospirosis.[218] This study has attempted to describe the basis of different diagnostic techniques with special emphasis on comparative analysis of different diagnostic tests. MAT was considered as the Gold Standard to determine the efficacy of different test for diagnosis of leptospirosis.

in our study different serological and molecular diagnostic tools such as latex agglutination test, IgM-ELISA, Indirect hemagglutination assay, Indirect Immunofluorescence antibody test, microscopic agglutination test and Real time PCR were used for diagnosis of human and animal leptospirosis. Interpretation of all tests used in the study is described below.

In present study conducted on human samples the sensitivity and specificity of Latex agglutination test was found to be 100% and 18.18% respectively, when compared with MAT. C. Hull-Jackson et.al. reported the sensitivity and specificity of latex agglutination 88% and 98% respectively during evaluation of commercial available latex agglutination test at Barbados.[184] TMA Senthilkumar et al. from Chennai also observed the sensitivity and specificity of latex agglutination test as 89.70% and 90.45% respectively. In another similar study conducted by Regional medical research center (ICMR) Port Blair, Andaman and Nicobar Islands, the latex assay had a sensitivity of 73.5%, specificity of 81.8% during the first week of illness. The sensitivity, specificity during the second week of illness was 89.8%. 78.8% respectively. The test also gave positive results for 11%, 10% and 7% against sera collected from patients having enteric fever, hepatitis B and malaria.[219]

Our results for latex agglutination assay have shown concordance with study conducted at Hawaii for evaluation of eight rapid screening tests for diagnosis of acute leptospirosis showing specificity of 10%.[220] The specificity obtained in this study may not reflect the performance of the assay in clinical studies conducted in diverse locations. This is particularly true for a disease with a wide spectrum of symptoms like leptospirosis, because the differential diagnosis is extremely broad and geographically variable. One of

the reasons for lower specificity of LA1 in our study is higher false positive results and unavailability of paired sera for MAT.

An IgM ELISA was carried out on the serum samples for detection of current infection. The test gave sensitivity and specificity of 77.77% and 30% respectively. Vaishali dohe et al. from Pune, India reported the sensitivity and specificity of an IgM ELISA to be 90% and 89.3% respectively, when compared with MAT.[221] Evaluation of four serologic assays for leptospirosis in subject groups from an urban center with highly endemic transmission in Brazil had sensitivity and specificity of an ELISA, 79.2% and 95.0% during acute phase of illness.[222] In one of the similar study conducted at Barbados, West Indies; P. Cumberland et al. assessed sensitivity and specificity of an IgM ELISA using 2 acute phase specimens (A1 and A2) and the convalescent phase specimen (C) and it was found to be (52% and 95%), (89% and 98%) and (93% and 94%) respectively.[175]

However, our study have shown concordance with seroprevalence study conducted on risk groups in Kerala, India has shown similar observations showing sensitivity of 76.2% and specificity of 30.5%.²⁹ The reason for low specificity associated with ELISA is related to multiple factors. This includes the individual differences in eliciting IgG and IgM antibody immune response and low titres making it undetectable by an ELISA. Significant false negativity and false positivity associated with ELISA methods leading to low specificity predictive and negative predictive value. Our data demonstrated that both the latex agglutination test and ELISA were more sensitive than the MAT for early antibody detection in the course of the disease. This suggests that both assays are suitable for leptospirosis screening.

In our study 119 human sera tested using Indirect hemagglutination assay showed

specificity of 92% and 95% respectively while evaluation of indirect hemagglutination test for diagnosis of human leptospirosis.[223] Mary bajani et al. evaluated four commercially available rapid serological test for diagnosis of leptospirosis at CDC; showed sensitivity and specificity of 38.5% and 95.8% respectively. Our study showed concordance with study conducted by Paul levett et. al., who reported IHA sensitivity of 49%, when tested on acute sample taken on the day of admission of hospital.[171] There are several steps in the procedure that affect the sensitivity and specificity of the indirect hemagglutination assay. The concentration of sheep red blood cells and antigen to be coupled is definitely significant. With increased red cell concentration there is decreased sensitivity of test. Excess of antigen may result in nonspecific agglutination.[224] One of the other reasons for low sensitivity of IHA is that, the test uses single, broadly reacting antigens to detect the patient's immune response to the infecting leptospires. Because the prevalence of endemic leptospira serogroups varies from place to place, the sensitivity of the IHA should reflect the ability of the IHA antigen to react with the specific leptospire serovars frequently causing infection in a particular location.[172]

Indirect Immunofluorescence antibody test was performed on all 119 human sera, which showed sensitivity of 55.55% and specificity of 40.9%. Marisa kemapunmanus et al. reported sensitivity and specificity of 91.9% and 100.0% respectively, while evaluating four immunodiagnostic assays for human leptospirosis. Similar study conducted at Thailand on evaluation of different serological tests, IFA showed sensitivity and specificity of 92.5% and 95% respectively, when compared with reference test MAT.[202] A study conducted in Hawaii has shown lesser sensitivity for IFA in

the collection of the acute phase specimen greatly affects the sensitivity of the IFA. H. Appassakij et al. mentioned drawback of genus-specific leptospiral antibody test was the possibility of cross reaction with other spirochetal infection. The reason for this is undetermined but is probably related to common antigen present on these spirochetes.[225]

Real Time PCR was carried on DNA extracted from 119 human sera showed sensitivity of 22.22% and specificity of 51% respectively. Similar study conducted at Hyderabad, India by Ahmed et.al. on human sera collected between 5 to 10 days of illness showed sensitivity and specificity of 69% and 100% respectively, when compared against culture and serology.[226] In another similar study conducted by Regional medical research center (ICMR) Port Blair, Andaman and Nicobar Islands, PCR had a sensitivity of 93.8%, specificity of 91.0% during acute stage of the disease.[219] Piyada wangroongsarb et. al. developed PCR using primers of 16SrRNA and flabB gene gave a sensitivity and specificity of 80.0% and 96.2% respectively.[227] PCR showed a low sensitivity in comparison to MAT. The lower sensitivity of PCR (relative to serology) may be attributed to the lower number of leptospire in the blood of patients with less severe infections. Unavailability of paired sera and low rate of isolation might explain low sensitivity in our study.

It was found that latex agglutination test and ELISA method gave the highest sensitivity 100%, the IHA and IFA gave similar sensitivities of 55.55%, the Real time PCR was lower at 22.22%.

MAT is considered the gold standard for diagnosis of leptospirosis. The MAT test was carried out on all 119 human sera. Among them only 9 (7.56%) were positive by taking 1:80 as a significant titre. Considering 1:100 as a significant titre the rate of

41%.[228] Largest scale studies on the baseline titre of the region need to be done to work out the correct cut off. The low positivity rate in our study may be because most of the samples were from the first week of infection.

Levett et al. described the sensitivity of the MAT to be 35% for the first week samples. They also mentioned that 8.1 days are required for the MAT to become positive for the diagnosis.[171]

Cumberland et al. in their study have reported sensitivity of 30% for the MAT in the acute phase samples. However, in their study they had taken 1:800 as a significant titre. They also concluded that, in the acute phase of the illness, there is little correlation between IgM antibody titre and MAT titre.[175] In the study conducted by Vijayachari et al. the sensitivity of MAT was 16.4% in the acute phase samples. Author had also mentioned that, the usefulness of the test during the first week was limited.[229] Hence, we tried to evaluate usefulness of the rapid, ELISA and PCR for the diagnosis during the first week of infection.

Concerning the specificity of the tests used, the IHA method also gave the highest test result of 53.63%, Real time PCR and IFA gave specificities of 51% and 40.9% respectively, an ELISA and latex agglutination test gave lower specificities of 30% and 18.18% respectively.

A total of 60 serum samples from rodents were also detected for leptospirosis using different diagnostic tools. But we could only process 31 samples using MAT, comparison between two tests were made considering only 31 samples.

Diagnosis of rodent samples showed sensitivity of 42.85% and specificity of 100% with latex agglutination test. Study conducted on prevalence of leptospirosis in rodents and

amongst using macroscopic slide agglutination test gave sensitivity of 17.6%.[230] Indirect hemagglutination assay was also carried out, which showed 72.42% sensitivity and 100% specificity. Evaluation of IHA for detection of leptospiral antibodies in wild mammals at California, USA showed sensitivity and specificity of 85.5% and 77.8% respectively, while rodent sera gave sensitivity of 60%.[231] Indirect Immunofluorescence antibody test showed sensitivity of 85.71% and specificity of 100%. Serological survey conducted on rodents in Republic of Croatia showed 12.7% antibody prevalence against *leptospira*. [40] Real Time PCR showed 0% sensitivity and 95.83% specificity. G.F. Mgode et.al., reported sensitivity of 11%, while detecting leptospiral DNA in rodents and insectivores from Tanzania.[232] The reason behind the low sensitivity of PCR in our study that, the rodents carry particular strain of *leptospira* in their kidneys and may shed them with their urine for long time and even for their lifespan, without causing any illness to them [50] implying that detectable antibodies may not be present.

MAT is considered the gold standard for diagnosis of leptospirosis. The MAT test could be done only on 31(51.66%) out of 60 rodent sera. Among them only 7 (22.58%) were positive by taking 1:80 as a significant titre. Largest scale studies on the baseline titre of the region need to be done to work out the correct cut off for animal leptospirosis.

Study conducted at Thailand on survey of leptospirosis showed percent positivity of 4.8% (56/1164).[233] Priya CG et.al.from Madurai, India reported 7 out 12 (58%) field rats were positive by MAT.[39] Serological survey conducted at Republic of Croatia determined the antibodies to different serovars of leptospires in 12.7% of small rodents.[40]

It was found that for diagnosis of rodent samples Indirect Immunofluorescence antibody method gave the highest sensitivity 85.71%, the IHA and IFA gave 71.42% and 85.71%, the Real time PCR positivity was almost nil,

Concerning the specificity of the tests used, the latex, IHA and IFA method gave the highest test result of 100%, Real time PCR gave lower specificities of 95.83%.

Comparative analysis of different tools was also done for diagnosis of Leptospirosis in dogs with gold standard test MAT.

Latex agglutination test showed lower sensitivity of 37.5% and specificity of 85.7%. TMA Senthilkumar et al., reported sensitivity and specificity of 93.05% and 91.95% respectively with recombinant OmpL1 antigen based latex agglutination assay, when compared with MAT.[218] Indirect hemagglutination assay showed 100% sensitivity and 57.14% specificity. Similar study conducted at Barbados on canine sera using IHA showed 100% sensitivity and 95% specificity.[234] Indirect Immunofluorescence antibody test showed 85.71% sensitivity and 100% specificity. Similar study conducted on canine sample by M A Rad et.al, showed sensitivity of 31% using IFA.[235] Real Time PCR gave sensitivity and specificity of 0% and 100%. Christine Brange et.al., carried out PCR on non-frozen canine samples gave a positive signal in all 19 cases whereas the samples tested after freezing gave positive results in only 6 of the 19 cases, a decrease in sensitivity of more than 60%.[209]

From the failure to demonstrate leptospires in the blood of dogs, we may infer that the animals were carriers of leptospires without causing them illness or at the time of testing organisms may be present in kidney tubules or organisms may have cleared from blood stream, because of an early treatment. The higher is the renal carriage; the lower is the

well established between some strains of leptospires and some animal species. *Leptospira* forms micro colonies in lumen of the convoluted tubules of kidney, which do not cause damage to the epithelium in carrier animals. Such animals become lifetime or short time carriers of leptospires and shed organism regularly or intermittently in urine. From the negative results of urine PCR and isolation, we may infer that at the time of collection of urine sample, animal might not shed the organism.

MAT is considered the gold standard for diagnosis of leptospirosis. The MAT test was carried out on all 40 canine sera. Among them 8 (20%) were positive by taking 1:40 as a significant titre. Considering 1:80 as a significant titre the rate of positivity drop down to 7 out of 40 (17.5%). Largest scale studies on the baseline titre of the region need to be done to work out the correct cut off for animal leptospirosis. Pam bautilier et.al., reported 28.8% seropositivity, while carrying out serological survey in dogs.[237] Study of leptospirosis conducted on dogs in and around Bengaluru, India showed 8.57% positivity, when screened with MAT.[238] V. Vijayanand et.al., from Chennai, India reported significant MAT titre in 30 (62.5%) dog sera.[216]

It was found that for diagnosis of dogs samples Indirect hemagglutination assay gave the highest sensitivity 100%, the IFA and latex agglutination test gave 87.5% and 37.5%, the Real time PCR positivity was not observed as animal might be carrier for the disease or Concerning the specificity of the tests used the Real time PCR and IFA method gave the highest test result of 100%, latex assay gave lower specificities of 85.7% followed by Indirect hemagglutination assay gave lowest specificity of 68.75%.

Thus to conclude, the study highlights the importance of risk groups since they are more vulnerable for acquiring Leptospirosis. In our study we found that students, housewives and outdoor workers are the most affected, as our study population was belong to urban

area. Hence, it is necessary to create awareness among the risk group as precautionary measure to avoid Leptospirosis. An association between risk factors was seen from our study. A strong relation was observed between rainwater contact and leptospirosis and also with animal contact and recreational activities. Based on the associated factor identified, an effective intervention program in a high risk occupational group can be implemented. The risk factor identified support the view the prevention of leptospirosis needs be multifactorial; particularly in tropical areas where leptospires are ubiquitous and where outdoor activities bring along several risk factors for leptospiral infection. Therefore health education is of paramount importance and should stress the need to apply all available measures relevant to risk activities, eg. to wear footwear. The present study demonstrated relationship between seropositive leptospirosis with occupational and environmental factors as well as recreational activities.

From the presence of leptospires in urine sample from rodents we may infer that rodents are the source of infection. It can therefore be inferred that in this city there was frequent and widespread exposure to rodents, which is the primary reservoir for leptospirosis in the urban environment¹². This finding is further supported by the large proportion of seropositive individuals who had their highest MAT titres against serovar pyrogenes, which is the same serovar associated with the seropositive rodents and dogs in our study.

Seroprevalence of Leptospirosis in animals complimented the study of seroprevalence of humans, pyrogenes was the common serovar found in both animals (rodent and dog) as well as humans. From this finding, we may be able to infer that animals are the carriers of *leptospira*. In our study, the high rates of leptospiral infection among rats and dogs and the predominance of serovar pyrogenes suggests a likely role for the rat in transmission

test being easily available. In spite of its poor specificity and tendency to give false positive results in other febrile disorders, rapid tests such as the Rapid test and latex agglutination test are the methods of choice in an outbreak. The latex agglutination test is extremely simple and rapid to perform. The result can be read within 5 minutes, compared to 3 hours using the dipstick assay or more than 1 hour with an ELISA test. The aggregation of latex particle is not difficult to read, the examiner can perform the test without the need for training. Special equipment is not required. This is an interesting alternative test for diagnosis of acute human leptospirosis especially in small health care units or in developing countries where sophisticated equipment is lacking. This assay has several potential advantages, which includes its simplicity and probability, limited amount of generated biomedical waste, and prolonged shelf life at ambient temperatures ranging from 2°C to 45°C. This would be an appropriate test for use in field studies, particularly in an outbreak situation.

Significant false positivity and false negativity associated with ELISA method leads to low specificity as compared to MAT. However, ELISA is valuable in those laboratories that do not have the facilities to perform MAT because it requires both the expertise and the maintenance of a panel of live leptospires which is cumbersome. Therefore ELISA may be considered as a better option for routine use. The ELISA has the following disadvantages: (i) only a single antigen is used, namely the genus-specific antigen which is shared by pathogenic and saprophytic leptospires alike; (ii) since it is based on genus-specific antigen, the ELISA test does not give an indication of infecting serovar.[200]

Molecular diagnosis of leptospirosis offers a promising alternative to culture or serology. Conventional PCR assays have previously demonstrated sensitivity and specificity for detection of leptospires, including diagnosis of culture-negative cases. However, earlier

PCR assays amplified both pathogenic and non-pathogenic *Leptospira* serovars and required amplification of 2 distinct targets to detect all pathogenic leptospires.

The real-time PCR assay targets the LipL32 gene for the major outer membrane lipoprotein, which is confined to pathogenic strains of *Leptospira* spp. and appears to be an important virulence factor. This assay offers the additional advantages of real-time PCR; amplification is more rapid, amplified product is detected without the requirement for gel electrophoresis, and the risk of post amplification contamination is reduced.

The test can detect *Leptospiral* DNA (antigen) in clinical samples like serum, urine, aqueous humour. Real Time PCR can rapidly confirm the diagnosis in early phase of the disease when bacteria may be present before antibody titre is in detectable levels. Real Time PCR is most sensitive in initial disease phase but is less sensitive than the serological tests over the course of the disease. In conclusion, we demonstrate the practical use of real time PCR for definitive diagnosis of acute leptospirosis at an early stage when the clinical picture is often confusing and conventional diagnostic tests are negative.

Indirect hemagglutination assay is a quantitative assay for the quantification of anti-*Leptospira* antibody. The test has different advantages as; it does not require many resources like IFA, ELISA, PCR and MAT. The results can be read visually, in comparison against immunofluorescence assay. The test is simple and convenient and sensitized fixed cells may be stored for at least a year. The test does not require specialized equipment and could be performed in peripheral laboratories with little expertise. IHA may be suitable for use in diagnostic laboratories for screening sera from acutely ill patients. The test detects genus-specific antibody (IgM and IgG) and cannot be used for determination of the infecting serovar. Hemagglutination, particularly indirect

hemagglutination, is a sensitive method for detecting small amounts of antibody, as little as 0.05 µg/mL - and is often used to 'titre' antiserum (Ab).

Immunofluorescence is histochemical laboratory staining used for demonstrating the antibodies bound to antigen in tissues or circulating body fluids. This technique permits early diagnosis of the disease. In conclusion, immunofluorescence methods provides useful adjunct to light microscopy for reaching diagnosis of diseases. Indirect Immunofluorescence antibody test is not possible to perform at smaller laboratories as it requires technical expertise, expensive instrument like Immunofluorescence microscope and caution to interpret the test results.

Immunofluorescence assay have different advantages as, diagnosis can be notified in relatively short time whether suspected case is positive or negative. As IF antibodies disappear earlier than agglutinating antibodies, a positive IF test almost always means fresh infection. In this connection it should be stressed that the IFA test as described in this communication should only serve as diagnostic assays and not for epidemiological surveys. Slides with fixed antigen can be prepared once every 3 months or even be acquired from central reference laboratories. These advantages should enable every diagnostic laboratory in which fluorescent microscopic equipment available to take upon itself diagnosis of leptospirosis using IFA.

MAT is the cornerstone of serological diagnosis and classification of the organism. The test involves elaborate procedure of examining each serum dilution mixed with little multiple antigens. This limits their applicability when a large number of sera to be tested. The MAT is very specific, but has the following disadvantages: (i) facilities for culturing and maintaining live Leptospire are needed; (ii) the method is technically demanding and time-consuming, particularly when the panel is large; (iii) antibodies may not be

round with a serovar that antigenically resembles the absent causative serovar (the finding of no titre or low titre in the MAT does not exclude Leptospirosis in these circumstances, it is never possible to be sure that the panel is complete since new unidentifiable Leptospire may cause disease and for this reason, it is advisable to include a genus specific screening test, such as an ELISA using a broadly reactive antigen); (iv) the MAT cannot be standardized because live Leptospire are used as antigen. It is a well-known fact that portion of single sample of serum from suspected case of leptospirosis tested in a number of different laboratories; the agglutination titre may show variation. These divergences are partly technical and partly personal.

Hence, the Performance of this test should be demarcated for better assessment of disease burden of leptospirosis in India. Leptospirosis screening tests that are sensitive and specific early in the acute phase of illness would be of great benefit to patients and physicians. Conversely, poorly performing screening tests may adversely impact clinical management, because a false-negative screening test result might discourage physicians from further considering leptospirosis and appropriate treatment might not be initiated. We concur with Faine et al. that “better bedside tests are required” ones which can recognize leptospirosis “earlier than serological methods”.

*Summary
&
Conclusions*

In the present study an attempt was made to determine the risk groups and risk factors for leptospirosis in and around Mumbai region. Out of 119 samples, 28 (23.48%) were found to belong to the risk group for leptospirosis. The highest positivity for leptospirosis was 8.40% (10) seen amongst students, 5.04% (6) amongst outdoor workers, 5.04% (6) amongst housewives, 2.52% (3) amongst farmers, 1.68% amongst medical personnel and 0.84% [8] amongst sewage workers. In our study it was observed that from the risk group, students seem to be at the highest risk of acquiring leptospirosis followed by housewives and outdoor workers.

From this study, we observed that rain water contact was the most common risk factor showing percent positivity of 29.41% (35), followed by animal contact 16.81% (14) and recreational activities 1.68% (2). In one of the studies from Japan, the authors have reported leptospirosis cases during recreational activity.

Consequently to study the transmission dynamics of leptospirosis an attempt was done to determine the sources of infection of leptospirosis. All urine samples from rodents were negative for isolation, but a positivity of 41.66% (5/12) was observed using Real Time PCR performed on DNA extracted from urine sediment. All positive rodent urine samples were found to belong to Pathogenic species of *Leptospira*. Characterization was done using LipL32 primers using Real Time PCR.²⁶ Hence it may be inferred that rodents play a major role as vectors for leptospirosis.

In the present study, we also tried to isolate the organism from human and animal samples. The rate of isolation of organism from human blood samples was 4.0% (4/50). The rate of isolation of leptospirae from the culture has been described

contamination. However, no isolation was possible from any animal blood or samples.

Study was also carried out to determine the seroprevalence of *Leptospira* in humans. Different conventional and modern diagnostic tools were used for the study. A total of 99 (83.19%) samples were positive with latex agglutination test (LAT), (54.4%) samples were found to be positive with indirect hemagglutination assay (IHA), (58.82%) samples were found positive using Indirect Immunofluorescence antibody test and all samples were tested using Microscopic agglutination test (MAT) in which 9 (7.56%) were found to be positive by taking 1:80 as the significant titre. The most common serovar found was *L. pyrogenes* followed by *Icterohemorrhagiae*, *bataviae*, *pomona*, *tarrassovi* and *canicola*.

Seroprevalence in animals (rodent and dog) was also carried out to determine the presence of leptospirosis as an indicator of source of infection.

Prevalence of Leptospirosis in rodents showed 3 (5%) samples positive by agglutination test, 5 (8.33%) samples were found to be positive with Indirect hemagglutination assay (IHA), 6 (10%) samples were tested positive with Indirect Immunofluorescence antibody test and 2 (3.33%) samples were confirmed for the presence of leptospiral DNA with Real Time PCR. The MAT assay was performed on 31 samples out of 60 samples. Of those, 7 (22.58%) samples were positive by MAT test. The most prevalent serovar was *L. pyrogenes* (85.71%), followed by *Icterohemorrhagiae* (14.29%).

agglutination test. Positive reaction was seen in, 18 (45%) samples with Indirect hemagglutination assay (IHA), 18 (45%) showed positive immunofluorescence Indirect Immunofluorescence antibody test. No sample was found positive for leptospiral DNA. All 40 samples were processed with Microscopic agglutination test (MAT), of which 8 (20%) were found to be positive for the test. The most common serovar detected was *L. pyrogenes* (100%), followed by *L. Icterohemorrhagiae* (Mixed infection with pyrogenes).

This study has attempted to describe the basis of different diagnostic techniques. A special emphasis on comparative analysis of different diagnostic tests was done against the Gold Standard reference technique MAT to determine the efficacy of different tests for diagnosis of human and animal leptospirosis.

Concerning the sensitivity and specificity latex agglutination test and ELISA method gave the highest sensitivity of 100% and IHA method gave the highest specificity of 53.63% for diagnosis of human leptospirosis.

It was found that for diagnosis of rodent samples Indirect Immunofluorescence antibody method gave the highest sensitivity of 85.71% and the latex, IHA and MAT method gave the highest specificity of 100%.

For diagnosis of dogs samples for leptospirosis Indirect hemagglutination assay gave the highest sensitivity of 100%. Concerning the specificity of the tests used the time PCR and IFA method gave the highest test result of 100%.

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Prevention and Control of Leptospirosis.

Appendix

10 APPENDIX

1. PREPARATION OF MEDIA (EMJH Medium)

Stock Solutions:

Albumin fatty acid supplement stock solutions:

Reagents	Grams per 100 ml H ₂ O	Storage
CaCl ₂ 2H ₂ O + MgCl ₂ 6H ₂ O	1.0 each	- 20°C
ZnSO ₄ 7H ₂ O	0.4	- 20°C
CuSO ₄ 5H ₂ O	0.3	+ 40 C
Vitamin B12	0.02	- 20°C
Tween 80	10	- 20°C

Basal medium stock solution:

Reagents	Grams per 100 ml H ₂ O	Storage
NH ₄ Cl	25.0	- 20°C
Thiamine	0.5	- 20°C
Glycerol	10	- 20°C

Method of Preparation:

Albumin Fatty Acid Supplement:

1. Dissolve 10 gms of bovine serum albumin in 60 ml. sterile glass distilled water

3. Add 1.5 ml Calcium Chloride and Magnesium Chloride stock solution.
4. Add 1 ml Zinc Sulphate stock solution.
5. Add 100 µl Copper Sulphate stock solution.
6. Add 50 mg Ferrous Sulphate and 40 mg. Sodium Pyruvate
7. Add 1 ml of Vit.B12 Stock solution.
8. Add 12.5 ml Tween 80 stock solution.
9. Add 24 ml Sterile distilled water to make up final volume of 100 ml.
10. Adjust pH to 7.4 - 7.6 with 1 N NaOH
11. Filter through using a Millipore filter pore size 0.22 µm.

Basal Medium:

1. Dissolve 1 gm of Na₂HPO₄ and 300 mg of KH₂PO₄ and 1 gm of NaCl in glass distilled water.
2. Add 1 ml Ammonium Chloride stock solution
3. Add 1 ml Thiamine stock solution.
4. Add 1 ml Glycerol stock solution.
5. Add Glass distilled water to make up a total vol. of 900 ml.
6. Adjust pH to 7.4
7. Finally make the volume to 1000 ml by adding glass distilled water.
8. Autoclave at 121°C for 30 minutes.

Basal Medium:	9 parts = 900 m
Albumin Fatty Acid supplement:	1 part = 100 m

Total	1000 m

Enrichment: Add (rabbit) serum aseptically to the EMJH medium to a concentration of 1 - 4% (v/v).

Selective medium: Add 5-fluorouracil aseptically to the EMJH medium to a concentration of 0.01–0.04%.

Quality control

This is carried out as follows:

Check for the absence of saprophytic leptospires and other contaminants. The med left for 1 week at 37 °C and room temperature. When the medium becomes turbid, microscopically and if contamination is found, discard.

2. Fontana's staining

Stock solutions:

A. Fixative

Acetic acid	1 ml
Formalin (40% HCHO)	2 ml
Distilled water	100 ml

Phenol	1 gm
Tannic acid	5 gm
Distilled water	100 ml

C. Ammoniated silver nitrate

Add 10% ammonia to 0.5% solution of silver nitrate in distilled water until the precipitate just dissolves. Now add more silver nitrate solution drop by drop until the precipitate returns and does not re-dissolve.

3. Reagents for SDS-PAGE

a. Acrylamide Stock Solution (30%):

One gram of Bisacrylamide was added to 30 ml Distilled water and heated upto 50 °C to dissolve. After this, 29 g of acrylamide was added and the final volume made upto 100 ml. the solution was stored in brown bottle at 4°C.

b. Stacking gel Buffer pH 6.8, 0.5M:

Six grams of Tris was dissolved in 40 ml D/w. The pH was then adjusted to 6.8 with HCl and the volume was made upto 100 ml.

c. Resolving gel buffer pH 8.8, 3M:

36.3 gms of Tris was added to 48 ml of D/w. The pH of the solution was adjusted and then the volume was made upto 100 ml using D/w.

One gram SDS was added to the mixture containing 3 gms of Tris and 14.4 g glycine and the final volume was made upto 1000ml.

e. Gel Loading Buffer Preparation:

0.5 M Tris (pH 6.8)	1 ml
Glycerol	2 ml
b-Mercaptoethanol	1 ml
10 % SDS	4 ml
Bromophenol Blue (0.25 %)	2.5 mg
Distilled Water	1.5 ml

f. Stacking Gel (5% acrylamide) Preparation:

Ammonium per sulphate (APS)	40 µl from 10% st
Distilled Water	2.7 ml
Stacking Gel Buffer	0.5 ml from stock
Acrylamide (5%)	0.67ml from stock
10 % SDS	40 µl
TEMED	4µl

g. Resolving gel (10 % acrylamide) Preparation:

Ammonium per sulphate (APS)	100 µl from 10%
Distilled Water	4.0 ml
Resolving gel Buffer	2.5 ml from stock
Acrylamide (10%)	3.3 ml from stock

TEMED

4 μ l

h. Staining solution (Coomassie Brilliant Blue) Preparation:

0.25% Coomassie Brilliant Blue (CBB-R 250) was dissolved in Methanol:Glacial . Acid:D/w (1:1:8 ratio) and filtered and stored in amber colored bottle at temperature.

i. Destain I

10% Methanol	30 ml
10% Glacial acetic acid	30 ml
D/w	120 ml

j. Destain II

50% Methanol	150 ml
10% Glacial acetic acid	30 ml
2% Glycerol	6 ml
D/w	234 ml

4. Alsever's solution for collection of erythrocytes:

a. Weigh out, dissolve in distilled water, and q.s. to 1 liter:

- 20.5 g dextrose
- 8.0 g sodium citrate dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$)
- 4.2 g sodium chloride (NaCl)
- 0.55 g citric acid ($\text{C}_6\text{H}_8\text{O}_7$)

After thorough mixing, check pH = 0.1 plus or minus 0.1. Adjust pH with 1 N NaOH or 1 N HCl, if necessary. Sterilize by filtration through a membrane filter with 0.22 μ m pore size. Contamination is likely in this solution if it is not adequately sterilized.

Phosphate-buffered saline (0.01M), pH 7.2 and pH 6.4 (PBS):

Prepare stock 25 times concentrated (25X) phosphate buffer containing in 100 ml:
2.74 g dibasic sodium phosphate (Na_2HPO_4) and 0.79 g monobasic sodium phosphate monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)

To prepare PBS, mix and dissolve in deionized, distilled water, and q.s. to 1 liter:
40 ml of 25X phosphate buffer and 8.5 g of sodium chloride (NaCl)

After thorough mixing, check pH = 7.2 /6.4 (as per requirement) plus or minus 0.1. Adjust pH with 1 N NaOH or 1 N HCl.

Autoclave or filter to sterilize.

Physiological saline, 0.85% NaCl:

Prepare a 20X stock solution by dissolving 170 g of NaCl in deionized water q.s. for 1000 ml.

Sterilize by autoclaving at 121°C.

To prepare physiological saline, 0.85% NaCl, add 50 ml 20X stock solution to 950 ml de-ionized water.

Sterilize by autoclaving at 121°C.

Store opened physiological saline at 4°C for no longer than 3 weeks.

*List
of
Abbreviations*

ARF	Acute Renal Failure
AST	Antibiotic Sensitivity Test
AP-PCR	Arbitrarily Primed PCR
BSA	Bovine serum albumin
BMC	Brihan Mumbai Municipal Corporation
CDC	Centers for Disease Control and Prevention
CSF	Cerebrospinal fluid
CFT	Complement Fixation Test
CBB	Coomassie brilliant blue
CIEP	Counterimmuno electrophoresis
DGM	Dark ground microscopy
DGGE	Denaturant gradient gel electrophoresis
DNA	Deoxyribose Nucleic Acid
EMJH	Ellinghausen, McCullough, Johnson and Harris
ELISA	Enzyme linked immunosorbent assay
ESS	Erythrocyte-sensitizing substance
FAFLP	Fluorescent amplified fragment length polymorphism
HEA	Heat extracted antigen
HA	Hemagglutiation
ICMR	Indian Council of Medical Research
IFA	Indirect Fluorescent Antibody Test
IHA	Indirect Hemagglutination Assay

Lip a	<i>Leptospira</i> immunoglobulin-like protein
LPS	Lipopolysaccharide
LFT	Liver function test
MSAT	Macroscopic Slide Agglutination Test
MRSP	Mapped restriction site polymorphism
MCAT	Microcapsule Agglutination Test
MAT	Microscopic Agglutination Test
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MAbs	Monoclonal Antibodies
MLST	Multi locus sequence typing
MRDE	Municipal rat destruction establishment
OMPs	Outer-membrane proteins
PBS	Phosphate buffer saline
PFGE	Pulse-Field Gel Electrophoresis
PUO	Pyrexia of Unknown Origin
RAPD	Random Amplified Polymorphic DNA Fingerprinting
Real Time PCR	Real Time Polymerase Chain Reaction
RMRC	Regional Medical Research Centre
REP-PCR	Repetitive extragenic PCR
REA	Restriction enzyme analysis
RFLP	Restriction Fragment Length Polymorphism
RF	Rheumatoid Factor

SEL	Sensitized erythrocyte lysis
MSAT	Serovar specific macroscopic agglutination test
SRBC	Sheep red blood cells
SSCP	Single strand conformation analysis
SDS- PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SDS	Sodium dodecyl sulphate
ILS	The International Leptospirosis Society
UTs	Union Territories
WHO	World Health Organization

Synopsis

A SYNOPSIS OF THE THESIS
TO BE SUBMITTED TO THE UNIVERSITY OF MUMBAI
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN APPLIED BIOLOGY

TITLE OF THE THESIS : **STUDY OF SEROPREVALENCE
OF LEPTOSPIROSIS USING
CONVENTIONAL AND MODERN
DIAGNOSTIC TOOLS**

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STUDY OF SEROPREVALENCE OF LEPTOSPIROSIS USING CONVENTIONAL AND MODERN DIAGNOSTIC TOOLS

INTRODUCTION

Civilizations in most countries have depended on animal life for their existence in a major way. It is therefore not surprising that there is often sharing and shifting of infections between the two, and in most occasions, it is from animals to human. There are an estimated 1415 infectious pathogens of which 62% are known to be zoonotic in nature.¹ Leptospirosis, the infection caused by leptospire, is the most widespread zoonosis in the world. Leptospire are ubiquitous spirochetes. While the saprophytic species is a usual contaminant of surface waters, the primary habitat of the pathogenic species is the distal tubules of kidneys in rodents, the reservoir hosts and various other animal species. Pathogenic leptospire shed in the urine of carrier animals contaminate the environment and cause human and animal infection throughout the world.² Large number of outbreaks has been reported from several countries in recent times, namely, Latin America, Caribbean, Nicaragua, Brazil, South East Asia, USA, Malaysia and India, origin of which has been traced to animals. Thailand and Sri Lanka are known to be endemic with the highest incidence rate.¹

In India, leptospirosis is endemic in most urban areas, where epidemic outbreaks occur after flooding caused by heavy seasonal rainfall.³ Outbreaks of leptospirosis have been reported from coastal districts of India, such as Gujarat, Maharashtra, Kerala, Tamil Nadu, Andhra Pradesh, Karnataka and Andaman and Nicobar Islands from time to time.

leptospirosis cases have been reported from Goa and Orissa. Mumbai experienced unprecedented heavy rains and a severe outbreak of leptospirosis in mid 2005.¹

Currently available data suggest that leptospirosis is the most common zoonosis worldwide. Although the disease is endemic in rural areas of the world, transmission continues in developed countries as well, for example in the US (especially tropical Hawaii but also inner cities), Ireland and Germany. Annual incidence of the leptospirosis vary from 0.53 per 100000 population in tropical areas to 0.04 per 100000 population in developed countries such as Germany.⁵ Based on global data collected by the International Leptospirosis Society (ILS) surveys there are currently 350000 to 500000 severe cases of leptospirosis annually. However, this is probably a heavy underestimation and the true extent of leptospirosis remains unknown because worldwide surveillance is lacking.⁶ A multi-centric study in India showed that leptospirosis accounts for about 12.7% of cases of acute febrile illness attending hospitals.⁷

In a study conducted all over the country by the National Reference Centre, Regional Medical Research Centre (RMRC; Indian Council for Medical Research), Port Blair during the period 2000–2001, a sero-positivity rate ranging from 0 to 46.8% amongst all cases of fever was observed from various parts of India. The positivity rate was highest in South India at 25.6%. It was observed to be 8.3%, 3.5%, 3.1% and 3.3% in Northern, Western, Eastern and Central India, respectively. Fourteen Cases of leptospirosis were reported through out the year at majority of the hospitals. Thirty-two percent of urban slum children in Mumbai had acute leptospirosis during monsoon in one of the studies.⁸

Leptospire can survive for long periods in the renal tubules of infected animals, without causing illness.⁸ Leptospirosis is primarily a monsoon disease, which affects domestic and wild animals as well as humans. Rats are the most common source of infection for

considered a “dead end host”. Several epidemics of leptospirosis have been reported worldwide during the past century, whereas in India, leptospirosis is endemic in most of the Southern and Western parts, where outbreaks usually occur after flooding caused by heavy seasonal rainfall. Some important outbreaks of leptospirosis that were reported in India in the recent past by the World Health Organization (WHO), National Institute for Communicable Diseases (NICD) Delhi and the National Leptospirosis Reference Centre, RMRC, Port Blair, were in August 2004, affecting Valsad, Navsari and Surat district of Gujrat (WHO). A post dengue outbreak of leptospirosis was also reported in Kerala and Tamilnadu (July 2003) and in Mumbai in 2005, (WHO). Leptospirosis outbreaks also occurred in Orissa in October–November 1999 (ICMR), October 1997, Valsad and Surat in Gujrat (NICD), 1997 Kerala (WHO), Diglipura of north Andaman during October–November 1993 (ICMR).⁹

In India, the disease has been recorded in Cattle, Buffalos, Horses, Sheep, Goats and Dogs (Arora, 1977; Uppal and Singh 1982). Among the earliest reports of leptospirosis that have been recorded were in Chennai (Madras) in 1932 by Ayyar; Sawney and Saxena (1967) from Madhya Pradesh; Rajasekhar and Nanjiah (1971) from Mysore; Triapthi (1977) from Orissa; Verma (1982) from Punjab and Dwivedi (1988) from Uttar Pradesh.⁹ Limited work in India on rodents indicated four species i.e., Norway rat (*Rattus norvegicus*), house rats (*Rattus rattus*), lesser bandicoots (*Bandicoota bengalensis*) and larger bandicoot (*Bandicoota indica*) as associated with the disease.¹⁰ Rodents were first to be recognized as carriers of leptospirae. They are often incriminated as the source of infection to human beings. Although serovar icterohaemorrhagiae has been often associated with rodents, other serovars have also been isolated. Very high seroprevalence rate and carrier rates (90.9% and 82.9% respectively) were observed among

anti-leptospiral antibodies following isolation of leptospire from suspected patients from the suburbs of Chennai. Elsewhere, in Tamil Nadu anti-leptospiral antibodies (52.1%) were evident from the field rodents. While relatively low prevalence was observed among the rat population, yielding two isolates from Andaman and Nicobar archipelago.¹¹

In Tamil Nadu seroprevalence study has shown 57.47% positivity among domestic animals and 72.73% in wild animals in captivity and 37.03% in rodents with a percent positivity of 56.68%.¹² Leptospiral infection among cattle was first recorded in Russia. Cattle worldwide may be infected with serovars hardjibovis, pomona, grippityphosa. Infection among buffalos and deer is also similar to that in cattle. Studies on cattle leptospirosis were conducted in other countries such as Spain and Australia also. Alonso-Andicoberry et al. (2001) studied 762 dairy cattle belonging to herds in Spain using Microscopic Agglutination Test (MAT) with 11 leptospiral serovars as antigens. Leptospiral antibodies were detected in 8% of the cattle. A serosurvey of animal populations of Andaman and Nicobar Islands showed that about 40% of the cows and 26% of the bulls were seropositive. Ratnam et al. (1983) screened 40 cows in a village near Chennai following an outbreak of leptospirosis in cattle. Antibodies against leptospire were found in 68% of the cows. Pigs are commonly infected with serovars pomona, tarassovi, grippityphosa, bratislava, sejroe, icterohaemorrhagiae and canicola (Faine 1994). Adult non-pregnant infected pigs are usually symptom-free and become chronic carriers. Historically, leptospirosis was recognized as a disease of dogs because it was known in any other animals. canicola and icterohaemorrhagiae are the common serovars that infect dogs. Acute leptospirosis in dogs is known as Stuttgart disease.

leptospirosis in human beings and dogs in Chennai City. Following the outbreak, a serosurvey was conducted among humans and dogs. Seroprevalence was 50.5% in humans and 21.3% in dogs. *Leptospira* belonging to serovar icterohaemorrhagiae was isolated from a human patient and canicola from a dog.¹¹ The prevalence of *Leptospira* antibodies in dogs has varied among different countries: 21.3% in India (Venkatachandra and Nedunchellian, 1992) and 6.36% in Italy (Cerri et al, 2003) while the prevalence was 11% in Thailand (Tongkorn Meeyam, 2004).¹³

Most human infections occur in young adult men and children and result from occupational or environmental exposure. Epidemiological studies indicate that leptospirosis is commonly associated with certain occupational workers such as farmers, sugarcane workers, veterinarians, and animal handlers. Leptospirosis can also be transmitted through recreational activities such as hiking, picnicking, swimming and canoeing.⁸ A study conducted on risk groups in India showed that in a veterinary group, out of 42 people, 15 (35.71%) showed the evidence of leptospirosis of which 10 were livestock workers, two each were veterinary doctors and attendants and one lab technician.¹⁴

Today, the disease is widespread in farm and domestic animals in many parts of India. Yet human leptospirosis is not commonly diagnosed in most of these places. It may have been under reported due to lack of awareness; its protean clinical manifestations and the unavailability of the laboratory tests.¹⁴ The present study was carried out to determine the prevalence of leptospirosis in human and animal using conventional and modern diagnostic tools.

Outbreaks of leptospirosis have recently occurred in various parts of India. Leptospirosis has now been recognized as an emerging infection. Human leptospirosis epidemic has occurred increasingly and continuously from many years in India. This can be used to hypothesize that the source of leptospirosis infection was continuous and circulate in the outbreak areas. The source of infection could have been carrier animals that shed *Leptospira spp.* or the environment that may be contaminated with leptospires. Therefore, the objective of this study was to determine the possible source of leptospirosis infection including carrier animals (i.e. dogs and rodents).

Risk factors for leptospirosis include living in rural areas and the epidemiology of leptospirosis reflects the ecologic relationship between humans and chronically infected mammalian reservoir hosts. Three study epidemiologic patterns of leptospirosis were originally defined by Faine et al. The first pattern is a "farming type" that occurs in temperate regions, due to direct contact with animals through exposure to a large number of serovars that infect cattle and pigs. The second pattern is an "urban type" which comprises rodent borne infection in the urban environment. The third pattern is a "tropical type" that occurs in humid, wet areas, from exposure to a larger number of serovars infecting a diversity of reservoir animals including rodents and farm animals. Recently, those considered at greater risk of contracting leptospirosis include farmers, pig farmers, butchers, agricultural workers, sewer workers along with those who participate in or supervise water-based recreational activities such as canoeing, swimming and sailing. The data generated during study of different risk groups will be helpful for applying preventive measures focusing a particular risk group.

Mumbai region. However, true incidence and prevalence of leptospirosis in Mumbai is unknown. In this study we made an attempt to determine the seroprevalence of leptospirosis in humans and animals, as it will provide information about circulating serovar in the study area. The disease has also been a diagnostic enigma for microbiology laboratories across the country. Since isolation rate of the causative organism from clinical specimens is low due to prior indiscriminate use of antibiotics and also due to difficult and expensive isolation techniques, currently, serological techniques remain the cornerstone of diagnostics. Various serological tests like Latex Agglutination Test, ELISA, Immunofluorescence Antibody Test (IFA), Microscopic Agglutination Test (MAT) and Indirect Hemagglutination Test (IHA) have been used. Microscopic Agglutination Test is considered to be the gold standard test; however, microbiology laboratories rarely perform it, since it requires considerable laboratory infrastructure, skilled technicians and is extremely labour intensive. Many serological tests like IgG and IgM ELISA are commercially available. Newer methods such as PCR and Real-time PCR are also proving their importance.

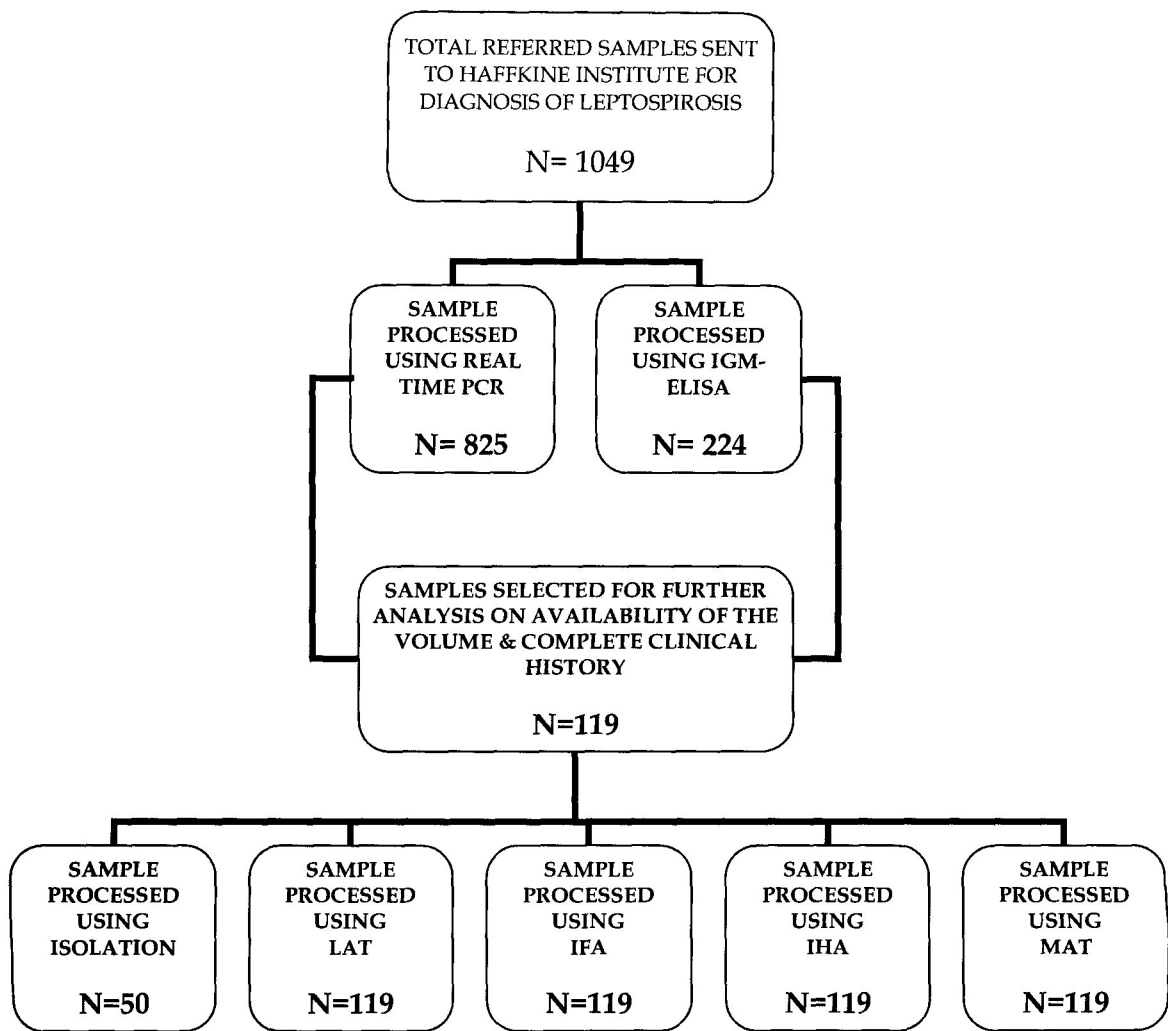
Hence, in our study, we compared different conventional and modern diagnostic methods for the diagnosis of leptospirosis. This will provide several promising approaches to the diagnosis of leptospirosis in future.

AIMS AND OBJECTIVES

- To study different risk groups, risk factors and sources of infection for the presence of leptospirosis.
- To study the prevalence of leptospirosis in humans and animals.
- Characterization of *leptospira* isolates to species level.
- Assessment of efficacy of different diagnostic tools used for diagnosis of leptospirosis.

MATERIAL AND METHODS:

Figure 29: Schematic flow diagram of human study subject categorization and leptospirosis testing



Where,

LAT=Latex agglutination test.

IFA=Indirect Immunofluorescence antibody test

IHA= Indirect hemagglutination assay.

MAT=Microscopic agglutination test,

1. BLOOD SAMPLES:

A: Human samples:

During 2006–2008, a total number of 1049 referred samples from the patients suspected of leptospirosis (as per WHO guidelines for diagnosis of Leptospirosis)¹⁵ were sent to the Nodal Laboratories for Re-emerging Diseases (viz. Leptospirosis, Dengue, Malaria, etc.) in Department of Zoonosis at Haffkine Institute, Mumbai from various hospitals in and around Mumbai region. Out of 1049 samples, as per the request of the referring doctors, Real-Time PCR was carried out on 825 samples and IgM ELISA was performed on 224 samples.

A total of 119 coded samples were selected for further analysis from 1049 samples on the basis of maximum volume of the samples available (as most of the samples sent to the centre were less in volume) and availability of complete clinical history of the patient.

Ten blood samples were collected from healthy individuals to serve as negative controls. All of them confirmed to be negative for anti-*leptospira* antibodies by an IgM-ELISA for leptospirosis. These samples were used as negative control for some of the diagnostic assays. Informed consent was obtained from the control group.

Sample processing:

Serum from the referred blood samples was separated from the plain bulb, aliquoted and stored at –20°C till further use. Whole blood was used for inoculation into EMJH medium for isolation of organism.

----- samples.

In this study, 60 rodents were included. Rodents were caught live from different regions from in and around Mumbai by rodent handlers of the Brihan Mumbai Municipal Corporation (BMC) and were brought to Municipal Rat Destruction Establishment (MRDE) at Haffkine Institute.

Blood samples were collected from 40 dogs brought for the treatment to Bombay Veterinary College, Parel, Mumbai with the symptoms of anorexia, fever, vomiting, dehydration and icterus.

Sample processing:

Rodents were characterized to species level and anaesthetized with anaesthetic ether, euthanized by cervical dislocation. Blood was withdrawn by heart puncture and collected in plain bulb. The serum was separated from the plain bulb, aliquoted and stored at -20°C till further use. Whole blood was used for inoculation into EMJH medium for isolation of organism.

Blood from dogs were withdrawn from cephalic vein with the help of veterinary doctors and collected in plain bulb. The serum was separated from the plain bulb, aliquoted and stored at -20°C till further use. Whole blood was used for inoculation into EMJH medium for isolation of organism.

2. URINE SAMPLES:

A total of 12 urine samples were collected from rodents. Urine was withdrawn from aseptically using syringe and needle. Urine samples were diluted using Phosphate Buffered Saline for inoculation into medium for isolation of organism. The remaining urine samples were preserved using formalin for DNA extraction.

A total of 6 urine samples were collected from dogs. Urine was withdrawn from aseptically using catheters with the help of veterinarians. Urine samples were diluted using Phosphate Buffered Saline for inoculation into medium for isolation of organism. The remaining urine samples were preserved using formalin for DNA extraction.

1. Isolation of organism:

The culture medium used for isolation of the organism was the EMJH (Ellinghausen McCullough-Johnson-Harris) medium which was modified by the addition of serum.

To make the medium selective, 5-fluorouracil was added at a concentration of 100 µg/ml of medium.

i. Blood / Urine Culture

Inoculation:

One to two drops of the blood/diluted urine was added aseptically to 5 ml of medium.

The medium was kept in incubator at 37°C for approximately 48 hours.

Thereafter it was kept at room temperature and observed daily for turbidity, growth

ii. Culture examination:

Cultures were observed using Dark Field Microscopy.

Microscopic examination was done every 3rd, 7th and 10th day of inoculation.

Approximately 5–10 µl of the culture was taken on a thin glass slide. A thin cover slip was placed on the preparation. The preparation was observed under 20× and 40× magnification.

Typical motility of the *leptospira*.

Negative cultures were not discarded till 2 months of inoculation.

The presence of leptospire in the culture was further confirmed by DNA extraction and performing Real time PCR as discussed further.

iii. Fontana's staining:

To confirm the presence of organism in the culture Fontana's staining method was used. A smear was prepared from culture on the glass slide and fixed with fixative. The slide was washed with alcohol and dried by heating gently on a Bunsen burner. After mordant was added and heated. The slide was washed again using distilled water and dried. Finally slide was treated with ammoniated silver nitrate till film turned brown on heating. Slide was washed with distilled water and mounted in paraffin and observed under oil immersion lens.

Leptospira appears dark brown colored against light pink background.

2. Latex Agglutination Test¹⁶:

Latex Agglutination Test kit was graciously provided by Regional Medical Research Centre (RMRC), Port Blair, Andaman and Nicobar islands. The kit was indigenously developed by RMRC.

Principle:

A latex agglutination assay is a rapid test for the detection of antibodies to leptospira using latex beads coated with the antigen.

The test was performed by placing 5 µl of serum sample on a glass slide. Subsequently the serum was mixed with 10 µl of coated latex particles. The slide was rotated gently for 2 minutes and read for the aggregation of the latex particles on the scale 0 to 3+. Positive and negative controls were included with every batch tested.

3. *Leptospira* IgM ELISA (Kit based)¹⁷:

ELISA test appears to be able to detect anti-leptospiral antibodies somewhat earlier than does MAT, beginning around 5 days after symptoms start.

To detect current infection, IgM antibodies were detected from the serum sample. The test was performed according to the manufacturer's instructions.

Principle:

The microwells are coated with purified *leptospira* patoc antigen. During the incubation with the patient's sera, any antibodies which are present will bind to the antigen coated on to the wells. After washing to remove the rest of the un-conjugated antibodies, the Enzyme conjugate is added. If antibodies have been bound to the antigen, the Enzyme conjugate will then bind to these antibodies. After another series of washes, the chromogen (Tetramethyl benzidine; TMB) is added. If the Enzyme conjugate is present, the peroxidase will catalyse a reaction that consumes the peroxide and turns the chromogen from clear to blue. Addition of the stop solution ends the reaction and turns the blue colour to yellow. The reaction can then be read visually.

4. Indirect hemagglutination assay¹⁸:

Principle:

Indirect hemagglutination assay is a genus specific test where the broadly reactive antigen is coated on erythrocytes. This is a quantitative assay for the quantification of anti-*Leptospira* antibody. This test can be done in microtiter plates or tubes.

In this test, sheep erythrocytes were sensitized by heat extracted antigen from serotype E-patoc/ pomona. A volume of 25 µl phosphate buffer saline was added to all wells of microtiter plate. A volume of 25 µl test serum specimens were then added to the respective wells and serially diluted. After that, 25 µl of antigen sensitized red blood cells were added into the microtiter plate. The mixture was shaken immediately in the plate for 15–30 seconds and put in a moisture box to prevent evaporation. The plate was incubated at room temperature for one hour and the agglutination pattern was then read. Control cells (unsensitized red cells) were also tested to screen for non-specific reactivity.

5. Indirect Immunofluorescence Antibody Technique (IFA)¹⁹:

Principle:

The Indirect Immunofluorescence antibody test is a double-layered technique. The unlabelled antibody (sample) is applied directly to the antigen bound to a slide and then

...with a fluorescein conjugated anti-human globulin. The test is performed to detect specific antibody response within a serum sample.

In this test, antigens from serotype E-patoc, tarrasovi + pyrogene and pomona grown in EMJH medium. A drop of 10 µl of a suitable culture was placed on each circular areas of a microscopic slide, using a micropipette and was allowed to dry at room temperature. The antigen slide was fixed with acetone for 10 minutes and dried by evaporation at room temperature. The antigen IFA slide was covered with 10 µl of diluted serum (1:80) and incubated in a moist chamber at 37°C for 30 minutes. The slide was washed three times with fresh PBS (pH 7.4) and air-dried. Finally the slide was treated with 10 µl of FITC-conjugated antihuman globulin Ig (G+M) (1:100) and incubated in a moist chamber at 37°C for 30 minutes. The slide was washed three times with PBS and mounted with glycerol buffer. Fluorescence microscopy was conducted with a fluorescence microscope under the 100× oil immersion lens. A positive result showed fluorescent green coloured complexes.

6. Real Time PCR²⁰:

Principle:

Real-Time Polymerase Chain Reaction is a laboratory technique based on the polymerase chain reaction, which is used to amplify and simultaneously quantify a targeted nucleic acid molecule. It enables both detection and quantification of a specific sequence in a sample.

i. Extraction of DNA

DNA was extracted from 100 µl of serum/urine sediments using DNA Sorb (Saccace biotechnologies, Italy) according to the manufacturer's guidelines. samples/urine sediments were mixed with lysis solution. Samples were then centrifuged and tubes were kept at 65°C for 5 minutes. A volume of 20 µl of sorbent was added to the supernatant, vortexed and centrifuged. Further supernatant was discarded and pellet was washed once with wash buffer 1 and twice with wash buffer 2. The tubes were kept at 65°C for 5 minutes with open lid. Finally 50 µl of DNA eluent was added to elute DNA. The extracted DNA was used for the amplification of leptospiral DNA using Real Time PCR.

ii. Real Time PCR:

Real Time PCR assay was performed using TaqMan chemistry in a volume of 25 µl reaction mixture (Primer Design, UK). The reaction was carried out on a Smart Real Time PCR Instrument (Cepheid, USA). Primers used in this study were Membrane protein LipL32 Primers, especially for the detection of pathogenic *leptospira*. The amplification conditions were initial denaturation at 95°C for 10 minutes, followed by 50 cycles of denaturation at 95°C for 10 seconds, annealing and extension at 60°C for 60 seconds.

Fluorogenic data for the amplified DNA was collected during the reaction through FAM channel.

7. Microscopic agglutination test:²¹

One hundred and nineteen human and one hundred animal (rodents and dogs) samples were sent to the RMRC reference laboratory, Port Blair, Andaman and Nicobar Islands as these tests require laboratory infrastructure dedicated for *Leptospira* culture, reference serovars and technical expertise.

Principle:

Microscopic agglutination test (MAT) is a standard serology agglutination test, using *Leptospira* serovars in equal volumes to dilutions of a serum sample. Results are given in titres of antibodies. The test will work with any species of mammalian serum. It is the gold standard Leptospirosis test. MAT measures IgM immunoglobulin rather than IgG as in ELISA and this enables MAT to give a better indication of recent infection. MAT is the definitive reference test on serum for pathogenic *Leptospira*s. Cross reactions between sera groups and serovars are common and the highest tittered serovar is taken as the infecting organism.

All 96 wells of microtitre plate were filled with 50 µl PBS. Another 140 µl PBS was added to the wells of column 2. A volume of 10 µl of test serum was added to the first well of column 2 (dilution 1:20) mixed and 100 µl was discarded. Serum was serially diluted by pipetting 50 µl from one well to the next and the final 50 µl was discarded (2->

wells. The plate was mixed thoroughly on a micro shaker and incubated for 2 hours at 30°C. For observation, one drop of mixture was transferred with a pipette from the well to a microscopic slide and examined under dark field microscope with 20× objective without cover slip.

The titre is the dilution that gives 50% agglutination, leaving 50% of cells free. If agglutination occurs with more than one serovar then the serovar giving highest titre taken as infective serovar. Test samples were compared with a control suspension of leptospire diluted 1:2 in PBS without serum.

The *leptospira* antigen battery used for the tests was as follows:

australis, bankinang, ballum, canicola, grippityphosa, hebdomadis, icterohaemorrhagiae, hardjp, pomona, pyrogenes, bataviae and tarrasovi.

Statistical Analysis:

The entire tests that were carried out for this study were compared with the reference technique using statistical analysis parameters as follows²²:

Sensitivity (%): $\frac{\Sigma TP \times 100}{\Sigma TP + \Sigma FN}$

Specificity (%): $\frac{\Sigma TN \times 100}{\Sigma TN + \Sigma FP}$

Positive predictive value (%): $\frac{\Sigma TP \times 100}{\Sigma TP + \Sigma FP}$

Negative predictive value (%): $\frac{\Sigma TN \times 100}{\Sigma TN + \Sigma FN}$

The present study was carried out to evaluate risk group and risk factors to determine prevalence of leptospirosis. Different risk groups and risk factors individuals that were found during this study are mentioned in Table 1. In this study percent positivity by different risk group (occupational) population was 23.53% (28). Many risk factors were associated with leptospirosis. In our study, the percent positivity of acute leptospirosis due to water contact was 29.41% (35). Leptospirosis acquired because of contact with animal shown percent positivity of 11.76% (14). Recreational activities seem to be the cause of leptospirosis in our study with percent positivity of 1.68%. Exposure due to different risk group and risk factors are shown in figure no.2.

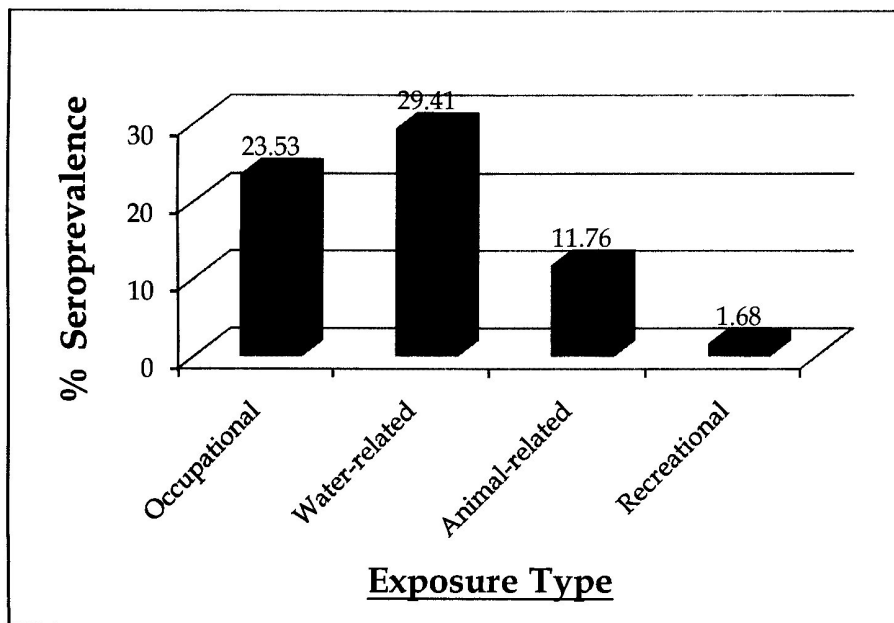
Leptospirosis

Characteristics		No. (%)
AGE (YEARS)	<25	38 (31.93 %)
	25-50	68 (57.14 %)
	>50	13 (10.94 %)
GENDER	Male	85 (71.43 %)
	Female	34 (28.57 %)
EXPOSURE TYPE	Occupational	28 (23.53 %)
	Water-related	35 (29.41 %)
	Animal-related	14 (11.76 %)
	Recreational	2 (1.68 %)
SYMPTOMS	Flu-like illness	33 (27.73 %)
	Headache	54 (45.38 %)
	Myalgia	65 (54.62 %)
	Pyrexia	96 (80.67 %)
	Lethargy	5 (4.20 %)
	Malaise	7 (5.88 %)
	Vomiting	34 (28.57 %)
	Diarrohea	5 (4.20 %)
	Abn. LFT's	3 (2.52 %)
	Jaundice	30 (%)
	Hepatic failure	5 (4.20 %)
	Renal failure	16 (%)
	ARDS	3 (2.52 %)
	Anorexia	1 (0.84 %)
	Dyspnoea	29 (%)
	Cough	2 (1.68 %)
	Thrombocytopenia	1 (0.84 %)
	Abortion	1 (0.84 %)
SIGNS	Conjunctivitis	11 (9.24 %)
	Hemoptysis	5 (4.20 %)
	Bleeding	2 (1.68 %)
	DIED	1 (0.84 %)

Occupational: Student-10, Housewife-6, Medical-2, Sewage worker-1, Farm Outdoor-6.

Water related: Rain water-19, Sewage-4, Pond-2, River-1, Canal-1, Farming-Unknown-7.

Animal related: Unknown-7, Farm live stock-3, Rats-2, Dogs-2.

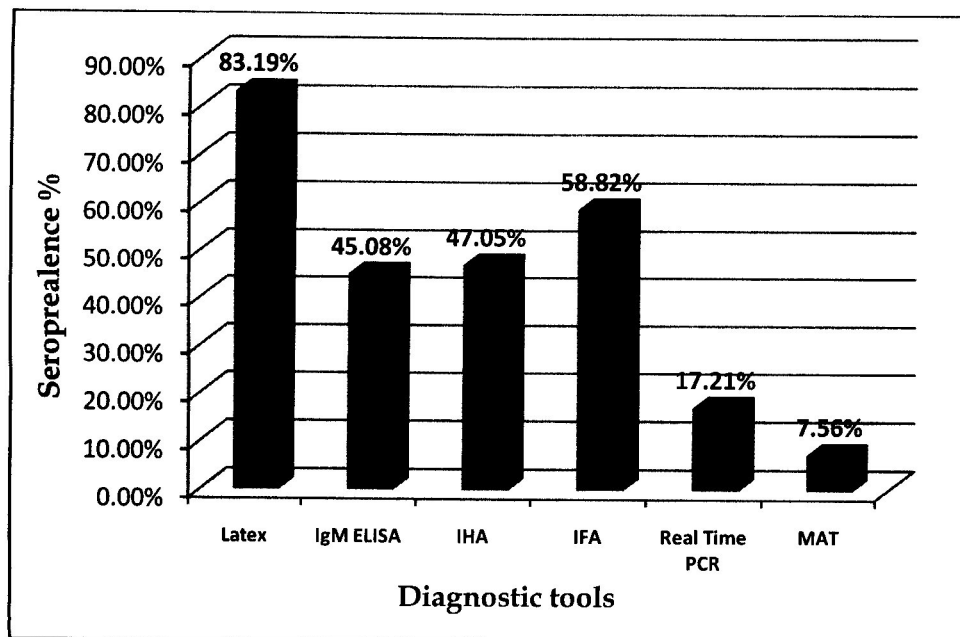


Leptospirosis in different risk group was analyzed using different diagnostic tool percent positivity shown by different tools are categorized into Figure 2. Out of 2 group samples, 23 (82.14%) were found to be positive with latex agglutination to (64.29%) samples were positive for IgM antibodies against *leptospira* using IgM I method, 14 (50%) samples were positive with Indirect hemagglutination assay (IH (50%) samples were positive with Indirect Immunofluorescence antibody test (IF, 15(53.57%) were positive for leptospiral DNA using Real Time PCR. All of then negative by Microscopic agglutination test (MAT).

samples from dogs were collected and cultured. 12 urine samples from rodents were found to be negative for isolation; however, 5 (41.66%) of them were found to be positive with Real Time PCR performed on DNA extracted from urine sediment. All urine samples from dogs were negative by both Real Time PCR and culture techniques. All positive samples from rodents were found to contain DNA from pathogenic *leptospira* confirmed by using Outer membrane protein LipL32 primers that can only identify pathogenic *leptospira* using Real Time PCR.

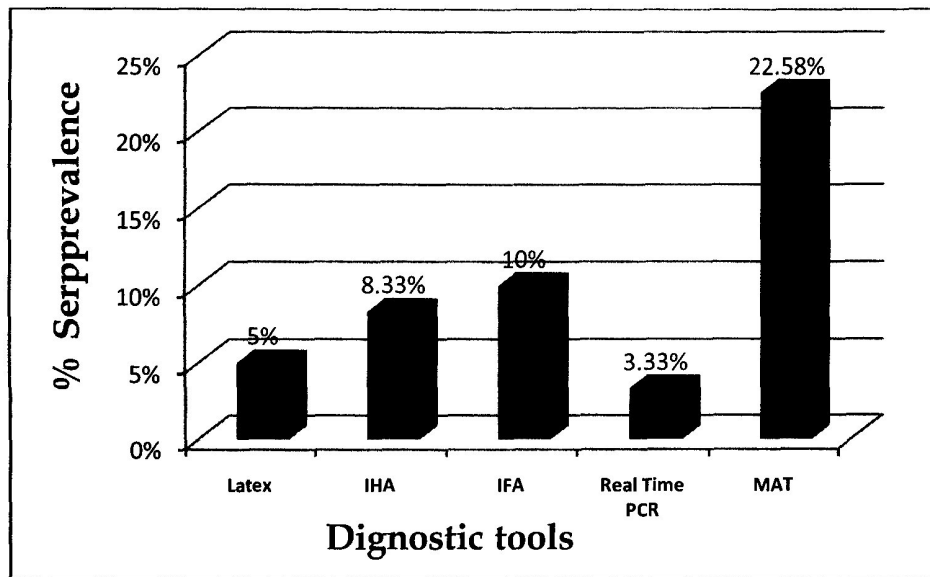
Prevalence of leptospirosis in human as well as animal in and around Mumbai region was one of the objectives of our study.

1. A) Seroprevalence of leptospirosis in humans was carried out using different diagnostic tools. Out of the referred 1049 samples, Real Time PCR was performed on 825 samples, of which 142 (17.21%) were positive for the test. ELISA was performed on 224 samples, of which 101 (45.08%) were positive. Out of 119 samples, 99 (83%) samples were positive with latex agglutination test. Indirect hemagglutination confirmed 56 (47.05%) samples for presence of anti *leptospira* antibodies. A total of 70 (58.82%) samples were found positive using Indirect Immunofluorescence assay test (IFA). Using Microscopic agglutination test (MAT), 9 (7.56%) were found positive for agglutinating antibodies. (as shown in fig.3) In 2 samples, mixed infection with different serovars was observed. The most common serovar found was pyrogenica followed by icterohemorrhagiae, bataviae, pomona, tarassovi and canicola.

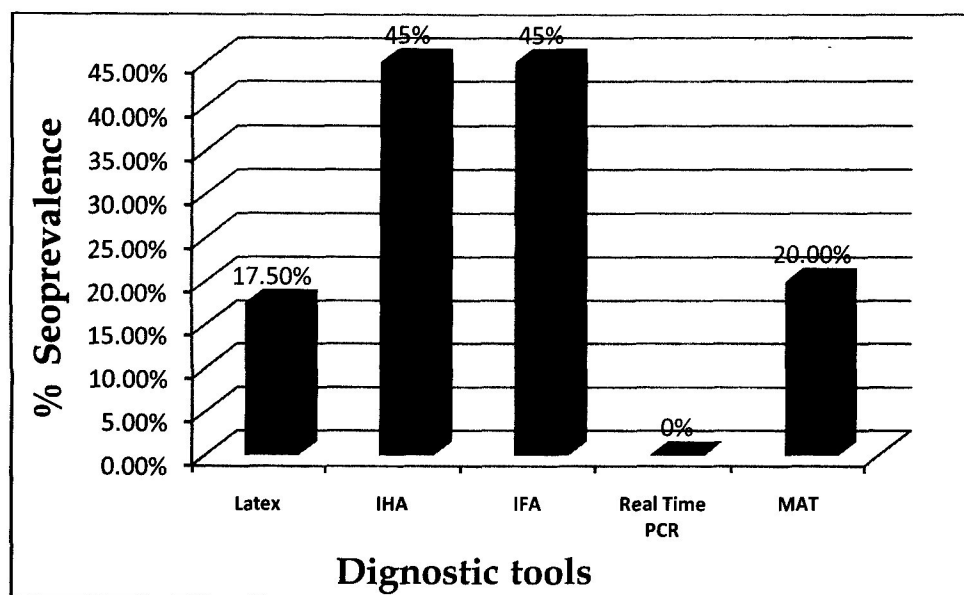


Seroprevalence study was also conducted in animals such as rodents and dogs.

1. B) A total of 60 blood samples were collected from the live rodents caught in different wards of Brihan Mumbai Municipal Corporation (BMC) with the help of staff. All 60 samples were tested with latex agglutination test and 3 (5%) were positive for the test. Indirect hemagglutination assay (IHA) showed positivity for 5 (8.33%) samples, while 6 (10%) samples were tested positive with Indirect Immunofluorescence antibody test (IFA). In 2 (3.33%) samples leptospiral DNA was detected using Real Time PCR assay. Out of 60 samples only 31 were processed with Microscopic agglutination test (MAT) as rest of the samples were spilled during transportation while sample being sent to RMRC, Port Blair, Andaman and Nicobar Islands. Out of 31 samples (22.58%) samples were positive for the test. The most common serovar detected was pyrogenes, followed by icterohemorrhagiae. Seroprevalence in rodents detected using different tools is shown in figure 4.



1. C) A total of 40 blood samples were collected from dogs suspected of leptospira. All samples were tested with different diagnostic tools for diagnosis of leptospira. A total of 7 (17.5%) samples showed positivity with latex agglutination test, Anti-leptospira antibodies were detected in 18 (45%) samples with Indirect hemagglutination (IHA) and when tested against Indirect Immunofluorescence antibody test (IFA) (45%) samples were found to be positive. All of the samples were negative for the presence of *leptospira* DNA. All 40 samples were processed with Microagglutination test (MAT), of which 8 (20%) were found to be positive (as shown in Figure 5) for the test. The most common serovar detected was pyrogenes, followed by icterohemorrhagiae. (Mixed infection)



Leptospira isolation allows definitive diagnosis of infection. One of the objectives study was to isolate the organism from blood and urine samples.

In this study 50 blood samples from humans suspected of leptospirosis were inoculated into EMJH medium. Out of them 4 (8%) were positive for isolation. The cultures were confirmed to be leptospires by morphology, Fontana's staining and extraction of DNA from all 4 samples and its amplification on the Real time PCR.

The characterization of the isolate to species level was one of the motives. All isolates were differentiated to their species level using Outer membrane protein LipL32 primers which can detect only pathogenic *leptospira* using Real Time PCR assay. All the isolates were belonging to pathogenic *leptospira*.

All blood samples collected from dogs and rodents were inoculated into EMJH medium. However, no isolation was possible from any of them. Twelve urine samples from rodents and six urine samples from dogs were diluted and inoculated into EMJH medium. All of them found to be negative for isolation.

for diagnosis of leptospirosis in humans and animals.

1. A) A total of 119 samples from humans were analyzed using different diagnostic Latex agglutination test were carried out for all of them, positive agglutination observed in 99 samples. To determine performance of latex agglutination test for diagnosis of leptospirosis, it was compared with reference technique, MAT. The results are shown in the table 2.

Table 2: Comparison of Latex Agglutination Test with MAT (Humans)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Latex Agglutination Test	Positive	9	90	99
	Negative	0	20	20
	Total	9	110	119
Index		Value		
Sensitivity		100%		
Specificity		18.18%		
Positive Predictive Value		9.09%		
Negative Predictive Value		100%		

were compared with gold standard test MAT to check efficacy of the test. The results are shown in table 3.

Table 3: Comparison of ELISA with MAT (Humans)

		Std. Criteria (MAT)		Total
		Positive	Negative	
ELISA	Positive	7	77	84
	Negative	2	33	35
	Total	9	110	119
Index		Value		
Sensitivity		77.77%		
Specificity		30%		
Positive Predictive Value		8.33%		
Negative Predictive Value		94.28%		

compared with standard criteria MAT. The result of comparative study was shown in Table 4.

Table 4: Comparison of Indirect Hemagglutination Assay with MAT (Humans)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Indirect Hemagglutination Assay	Positive	5	51	56
	Negative	4	59	63
	Total	9	110	119
Index		Value		
Sensitivity		55.55%		
Specificity		53.63%		
Positive Predictive Value		8.92%		
Negative Predictive Value		93.65%		

presence of antibodies against *leptospira*. Positive Immunofluorescence was found out of 119 samples. The test results were compared with standard test MAT. The results are shown in Table 5.

Table 5: Comparison of Indirect Immunofluorescence Antibody Test with (Humans)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Indirect Immunofluorescence Antibody Test	Positive	5	65	70
	Negative	4	45	49
	Total	9	110	119
Index		Value		
Sensitivity		55.55%		
Specificity		40.9%		
Positive Predictive Value		7.14%		
Negative Predictive Value		91.83%		

DNA. To determine the efficacy of the test, it was compared with MAT. The results shown in Table 6.

Table 6: Comparison of Real Time PCR with MAT (Humans)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Real Time PCR	Positive	2	54	56
	Negative	7	56	63
	Total	9	110	119
Index		Value		
Sensitivity		22.22%		
Specificity		50.9%		
Positive Predictive Value		3.57%		
Negative Predictive Value		88.88%		

using different diagnostic tools. But as only 31 samples were processed using comparison between two tests was made using 31 sample numbers.

Latex agglutination test was used to detect the anti-*leptospira* antibodies. Only 31 samples were found to be positive for the antibodies. To assess the efficacy of tl it was compared with reference technique MAT. The results are shown in Table 7.

Table 7: Comparison of Latex agglutination test with MAT (Rodents)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Latex Agglutination Test	Positive	3	0	3
	Negative	4	24	28
	Total	7	24	31
Index		Value		
Sensitivity		42.85%		
Specificity		100%		
Positive Predictive Value		100%		
Negative Predictive Value		85.71%		

evaluated using gold standard test MAT. The results are shown in table 8.

Table 8: Comparison of Indirect hemagglutination assay with MAT (Rodents)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Indirect Hemagglutination Assay	Positive	5	0	5
	Negative	2	24	26
	Total	7	24	31
Index		Value		
Sensitivity		71.42%		
Specificity		100%		
Positive Predictive Value		100%		
Negative Predictive Value		92.3%		

showed positive immunofluorescence. The efficacy of the test was determined by comparing it with standard technique MAT. The results are shown in table 9.

Table 9: Comparison of Indirect Immunofluorescence antibody test with MAT (Roc

		Std. Criteria (MAT)		Total
		Positive	Negative	
Indirect Immunofluorescence Antibody Test	Positive	6	0	6
	Negative	1	24	25
	Total	7	24	31
Index		Value		
Sensitivity		85.71%		
Specificity		100%		
Positive Predictive Value		100%		
Negative Predictive Value		96%		

Leptospira spp. from rodent samples were detected using Real Time PCR technique. Only 1 out of 31 samples were found to be positive for the presence of Leptospiral DNA. The performance of the test was determined by comparing it with gold standard test MAT. The results are shown in table 10.

Table 10: Comparison of Real Time PCR with MAT (Rodents)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Real Time PCR	Positive	0	1	1
	Negative	7	23	30
	Total	7	24	31
Index		Value		
Sensitivity		0%		
Specificity		95.83%		
Positive Predictive Value		0%		
Negative Predictive Value		76.66%		

using conventional and modern diagnostic tools.

Latex agglutination test was carried out on all samples, 7 of them were found positive for the test. The performance of the test was evaluated by comparing it with reference test MAT. The results are shown in Table 11.

Table 11: Comparison of Latex agglutination test with MAT (Dogs)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Latex Agglutination Test	Positive	3	4	7
	Negative	5	28	33
	Total	8	32	40
Index		Value		
Sensitivity		37.5%		
Specificity		85.7%		
Positive Predictive Value		42.85%		
Negative Predictive Value		84.84%		

antibodies. Out of 40 samples, 18 were found to be positive for the test. The result compared with reference test to determine efficacy of the test. The results are shown in table 12.

Table 12: Comparison of Indirect hemagglutination test with MAT (Dogs)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Indirect Hemagglutination Assay	Positive	8	10	18
	Negative	0	22	22
	Total	8	32	40
Index		Value		
Sensitivity		100%		
Specificity		68.75%		
Positive Predictive Value		44.44%		
Negative Predictive Value		100%		



comparing it with standard technique MAT. The results are shown in table 13.

Table 13: Comparison of Indirect Immunofluorescence antibody test with MAT (D

		Std. Criteria (MAT)		Total
		Positive	Negative	
Indirect Immunofluorescence Antibody Test	Positive	7	11	18
	Negative	1	21	22
	Total	8	32	40
Index		Value		
Sensitivity				87.50%
Specificity				100%
Positive Predictive Value				100%
Negative Predictive Value				95.45%

Time PCR technique. The efficacy of the test was determined using reference technique MAT. The results are shown in table 14.

Table 14: Comparison of Real Time PCR with MAT (Dogs)

		Std. Criteria (MAT)		Total
		Positive	Negative	
Real Time PCR	Positive	0	0	0
	Negative	8	32	40
	Total	8	32	40
Index		Value		
Sensitivity		0%		
Specificity		100%		
Positive Predictive Value		0%		
Negative Predictive Value		80%		

In leptospirosis, risk groups are certain groups of humans in a population that are more likely to be exposed as a result of their occupational/recreational activities. New risk groups may be formed as a result of changes in agricultural or social practices, or in reservoir animal populations in an area. Also there are many factors such as rain water contact, animal contact and recreational activities responsible for the risk of acquiring leptospirosis.

In the present study an attempt was made to determine the risk groups and risk factors for leptospirosis. Out of 119 samples, 28 were found to belong to the risk group. The highest positivity was 8.40% (10) amongst students, 5.04% (6) amongst outdoor workers, 5.04% (6) amongst housewives, 2.52% (3) amongst farmers, 1.68% (2) amongst medical personnel and 0.84% [8] amongst sewage workers. In our study, it was observed that from the risk group, students seem to be at the highest risk of acquiring leptospirosis followed by housewives and outdoor workers. Leptospirosis surveillance carried out by Leptospirosis Reference Laboratory in Queensland and Australia shown similar positivity amongst farmers (2.7%) and lower positivity amongst students (4.5%) as compared to our study.[131] One of the reason of lower positivity in farmers is that the study population was primarily urban (study area).

We also made an attempt to determine the risk factors responsible for the leptospirosis. From this study, we observed that rain water contact was the most common risk factor showing percent positivity of 29.41% (35), followed by animal contact 11.76% (14) and recreational activities 1.68 (2). In one of the studies from Japan, the authors have reported leptospirosis cases during recreational activity.[51]

... attempt to determine the source of infection of leptospirosis. All urine samples from rodents were negative for isolation, but a positivity of 41.66% (5/12) was observed using Real Time PCR performed on DNA extracted from urine sediment. All urine samples from dogs were negative for both Real Time PCR and isolation techniques. Failure to demonstrate leptospires in the urine of dogs does not necessarily eliminate the possibility that the animal is a chronic renal carrier; it merely indicates that the animal was not excreting detectable numbers of leptospires at the time of testing.²⁵

All positive rodent urine samples were found to belong to Pathogenic species of *leptospira*. Characterization was done using LipL32 primers using Real Time PCR.²⁶ Hence, it may be inferred that rodents play a major role as vectors for leptospirosis.

The rate of isolation of organism from human blood samples was 8% (4/50). The rate of isolation of leptospires from the culture has been described to be very low due to various reasons including prolonged incubation or frequent media contamination. Prior antibiotic usage might have affected the isolation of organisms in our study as urban population was involved to a larger extent. A similar study by S Chandrasekaran et al. showed similar positivity (8.2%) of culture from Madurai, India.[212]

However, no isolation was possible from any animal blood or urine samples.

Isolates were characterized to species level using LipL32 primers by Real Time PCR assay. It was observed that all 4 isolates belong to pathogenic species.

One of the objectives of the study was to determine the seroprevalence of Leptospirosis in humans. Different conventional and modern diagnostic tools were used for the study. A total of 99 (83.19%) samples were positive with latex agglutination test, 68 (54.4%) samples were found to be positive with indirect hemagglutination assay, 70 (58.82%)

samples were tested using Microscopic agglutination test (MAT), of which 9 (7) were found to be positive by taking 1:80 as the significant titre. The most common serovar found was pyrogenes followed by icterohemorrhagiae, bataviae, potterii and canicola. A similar study conducted in Mumbai post deluge outbreak during 2005 shown presence of similar circulating serovars as per our findings.³

Seroprevalence in animals was carried out to determine presence of leptospirosis as an indicator of source of infection.

Prevalence of Leptospirosis in rodents showed 3 (5%) samples positive by agglutination test, 5 (8.33%) samples were found to be positive with Indirect Hemagglutination assay (IHA), 6 (10%) samples were tested positive with Indirect Immunofluorescence antibody test and 2 (3.33%) samples were confirmed for presence of leptospiral DNA with Real Time PCR. The MAT assay was performed on 60 samples. Of those, 7 (22.58%) samples were positive for the test as shown in Fig.4). The most prevalent serovar was pyrogenes (85.71%), followed by icterohemorrhagiae (14.28%).

Prevalence of Leptospirosis in dogs showed 7 (17.5%) samples prevalent with agglutination test. Positive reaction was seen in, 18 (45%) samples with Indirect Hemagglutination assay (IHA), 18 (45%) showed positive immunofluorescence with Indirect Immunofluorescence antibody test. No sample was found positive for leptospiral DNA. All 40 samples were processed with Microscopic agglutination test (MAT) of which 8 (20%) were found to be positive for the test (as shown in Fig.5). The common serovar detected was pyrogenes (100%), followed by icterohemorrhagiae (Mixed infection). Similar observation made in one of the studies on seroprevalence of leptospirosis in animals from Tamil Nadu, India.¹²

reference technique MAT.

Diagnosis of human samples with Latex agglutination test showed a sensitivity of 100% and specificity of 18.18%. Study conducted at Hawaii has shown low specificity of 10%.²⁸ IgM ELISA was also performed on the serum samples for comparison and sensitivity and specificity of 77.77% and 30% respectively was observed. Seroprevalence study conducted on risk groups in Kerala, India has shown similar observations showing sensitivity of 76.2% and specificity of 30.5%.²⁹ The reason for low specificity associated with ELISA is related to multiple factors. This includes the individual differences in eliciting IgG and IgM antibody immune response and low titers making it undetectable by an ELISA. Indirect hemagglutination assay showed 55.55% sensitivity and 53.63% specificity. Indirect Immunofluorescence antibody test showed sensitivity of 55.55% and specificity of 40.9%. A study conducted in Hawaii has shown lesser sensitivity for IHA and IFA as compared to our study (26% and 38%).²⁸ The time between the onset of symptoms and the collection of the acute phase specimen greatly affects the sensitivity of the IHA and IFA.

Real Time PCR showed sensitivity of 22.22% and specificity of 51%. PCR showed a low sensitivity in comparison to MAT. The lower sensitivity of PCR (relative to serology) may be attributed to the lower number of leptospire in the blood of patients with less severe infections. Unavailability of paired sera and low rate of isolation might explain low sensitivity in our study. The sensitivity shown by Latex agglutination test and ELISA for diagnosis of human sample was higher (100%) as compared to other tests. Real Time PCR showed highest specificity of 51%.

latex agglutination test. Indirect hemagglutination showed 72.42% sensitivity and specificity. Indirect Immunofluorescence antibody test showed sensitivity of 85.71% and specificity of 100%. Serological survey conducted on rodents in Republic of China showed 12.7% antibody prevalence against *leptospira*.³⁰ Real Time PCR showed 95.83% sensitivity and 95.83% specificity. The reason behind the low sensitivity of PCR that rodents carry particular strain of *leptospira* in their kidneys and may shed them with urine for long time and even for their lifespan, without causing any illness to them, implying that detectable antibodies may not be present. For diagnosis of rodent species, Indirect Immunofluorescence antibody test showed highest sensitivity of 85.71% and highest specificity of 100% compared to other test.

Comparative analysis of different tools was also done for diagnosis of *Leptospira* in dogs with gold standard test MAT.

Latex agglutination test showed lower sensitivity of 37.5% and specificity of 85.71%. Indirect hemagglutination assay showed 100% sensitivity and 57.14% specificity. Serological study at Barbados showed percent 100% sensitivity and 95% specificity.²⁹ Indirect Immunofluorescence antibody test showed 85.71% sensitivity and 100% specificity. Sensitivity shown by Real Time PCR test was 0% and specificity was 100%. From the failure to demonstrate leptospires in the blood of dogs, we may infer that the animals were carriers of leptospires without causing them illness or at the time of sampling, the organisms may be present in kidney tubules or organisms may have cleared from the system, because of an early treatment.

of 100% and Indirect Immunofluorescence antibody test showed highest specificity of 100% compared to other tests.

Thus to conclude, the study highlights the importance of risk groups since they are more vulnerable for acquiring Leptospirosis. In our study we found that students, housewives and outdoor workers are the most affected, as our study population was belong to urban area. Hence, it is necessary to create awareness among the risk group as precautionary measure to avoid Leptospirosis. An association between risk factors was seen from our study. A strong relation was observed between rainwater contact and leptospirosis and also with animal contact and recreational activities. From the presence of leptospires in urine sample from rodents we may infer that rodents are the source of infection. The risk factor identified support the view the prevention of leptospirosis needs be multifactorial; particularly in tropical areas where leptospires are ubiquitous and where outdoor activities bring along several risk factors for leptospiral infection. Therefore health education is of paramount importance and should stress the need to apply all available measures relevant to risk activities, eg. to wear footwear. Seroprevalence of Leptospirosis in animals complimented the study of seroprevalence in humans. Serovar pyrogenes was the common serovar found in both animals as well as humans. From this finding, we may be able to infer that animals are the carriers of *leptospira*. Incidence of leptospirosis is often under reported due to the lack of a single diagnostic test being easily available. In spite of its poor specificity and tendency to give false positive results in other febrile disorders, rapid tests such as the Rapid test and latex agglutination test are the methods of choice in an outbreak. Significant false positivity and false negativity associated with

requires both the expertise and the maintenance of a panel of live leptospires which is cumbersome. Therefore ELISA may be considered as a better option for routine use. Real Time PCR can detect Leptospiral DNA (antigen) in clinical samples like serum, urine, aqueous humour. Real Time PCR can rapidly confirm the diagnosis in early stages of the disease when bacteria may be present before antibody titre is in detectable range. Real Time PCR is most sensitive in initial disease phase but is less sensitive than serological tests over the course of the disease. This study showed that Real Time PCR can be used to detect leptospires in the acute stage of leptospiral infections. The ability of the PCR to identify acute cases has clinical value for management of the patient. We believe that Real Time PCR will be a valuable tool for early diagnosis of leptospirosis. Indirect hemagglutination assay gives more false positive results. Indirect Immunofluorescence antibody test is not possible to perform at smaller laboratories. It requires technical expertise and expensive instrument like Immunofluorescence microscope.

Microscopic Agglutination Test (MAT) is the cornerstone of serological diagnosis and classification of the organism. It is not available to all laboratories and several reference laboratories. Performance of this test should be demarcated for better assessment of disease burden of leptospirosis in India. We concur with Faine et al. that “better diagnostic tests are required” ones which can recognize leptospirosis “earlier than serological methods”.

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