

TO STUDY THE PREVALENCE OF LEPTOSPIROSIS IN
RODENT POPULATION IN MUMBAI AND NEARBY AREAS

THESIS SUBMITTED
UNIVERSITY OF MUMBAI

FOR THE DEGREE OF
MASTER OF SCIENCE
IN
APPLIED BIOLOGY

SUBMITTED BY
VIKRANT H. TRIPATHI

UNDER THE GUIDANCE OF
DR. B. D. SATHE

DEPARTMENT OF ZOONOSIS
HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
MUMBAI

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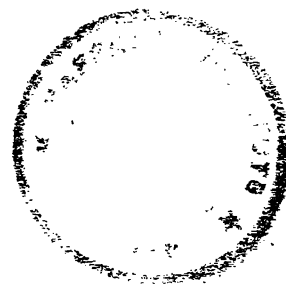
Mr. Vikrant H. Tripathi

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PAREL, MUMBAI – 400 012.**

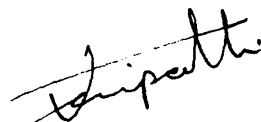


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Statement by the Candidate

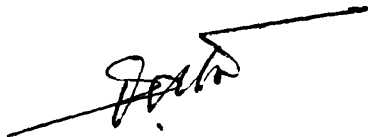
As required by the university regulation No. R2190, I wish to state that the work embodied in this thesis titled "To study the prevalence of leptospirosis in rodent population in Mumbai and nearby areas" forms my own contribution to the research work carried out under the guidance of Dr. B. D. Sathe at the Dept. Of Zoonosis, Haffkine Institute, this work has not been submitted for any other degree of this or any other university, Whenever reference have been made to previous works of others, it has been clearly indicated as such and included in the Bibliography.


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Dr. B. D. Sathe.

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DEDICATED TO MY PARENTS

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INTRODUCTION

INTRODUCTION

Leptospirosis is an ubiquitous zoonosis, caused by infection with a spirochete of the genus *Leptospira*. It is presumed to be the most widespread zoonosis in the world.⁽¹⁾⁽²⁾

The name *Leptospira* is derived from the greek language where 'Leptos' comes from greek word 'lepo' which stands for something fine and 'speira' in greek means fold or coils, so the name *Leptospira* fits their morphology.⁽³⁾

The source of infection is usually direct or indirect contact with the urine or contaminated materials like abortions, media, soil, fodder from an infected source. The incidence of the disease is significantly higher in warm climate regions than in temperate regions as the leptospires survive properly in warm, humid conditions. The disease can be termed as seasonal, as the incidence is at its peak during the monsoon and post monsoon period due to transmission of the organisms by the means of water in flooded areas and drains.⁽²⁾

Leptospiral infections have a very wide host range. It has been described in all types of warm blooded vertebrates. Mammals, marsupials and possibly birds can be infected in natural conditions, while all can be infected experimentally. All these can be divided into two groups i.e. maintenance hosts and accidental 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 including humans may become infected by indirect contact with maintenance hosts. Animals may be

maintenance hosts of some serovars but accidental hosts of others, infection with which may cause severe or fatal disease. The most important maintenance hosts are small animals like rodents, which may transfer infection to domestic farm animals, dogs and humans. The extent to which infection is transmitted depends on many factors, including climate, population density, and the degree of contact between maintenance and accidental hosts. Here the human to human transmission of the infection is rare but has been reported.⁽¹⁾⁽²⁾

The principles that govern the development, course and management of leptospirosis are the same in all animals. The clinical manifestations, severity and progress and epidemiological consequences of leptospirosis differ according to the nature of the infecting leptospire, and the biology, habits and habitat of the animal species. The risks and consequences of leptospirosis for humans are determined by sociogeographical considerations. It is especially hard to control transmission of leptospirosis to humans because it is a zoonotic disease affecting people whose lives are inextricably connected with wild or domesticated animals. Due to this reason it can be also termed as a disease of occupations. And those at a high risk of infection are people like farm workers, veterinarians, pet shop owners, field agricultural workers, milkers, plumbers, sewer workers, meat handlers, slaughter house workers, coal miners, workers of fishing industry, military troops, etc.⁽¹⁾⁽⁴⁾

There are many synonyms for leptospiral infections depending on the infecting serovar, severity and clinical manifestations of the infection, these are Weils disease, swineherds disease, canicola fever, canefield leptospirosis, swamp or mud fever and Indonesian Weils disease.⁽³⁾

Historical aspect :-

The classical description of leptospirosis is that of Weils disease, a dramatic acute febrile and sometimes epidemic illness characterized by jaundice, splenomegaly, often leading to renal failure. It was first reported over 100 years ago by Adolf Weil in Heidelberg. However, an apparently identical syndrome occurring in sewer workers was described several years earlier. Meanwhile the disease was confused with yellow fever when Stimson observed the spirochete by silver staining in a tissue section from kidney of a patient who was diagnosed with yellow fever but had probably died of Leptospirosis. The spirochete observed by Stimson has hooked ends, and so he named it as *Spirochaeta interrogans* due to their resemblance to a question mark. This confusion lasted till the time when the viral origin of yellow fever was established.⁽¹⁾⁽²⁾

Whereas in ancient China, leptospirosis was recognized as an occupational hazard of the rice harvesters and the Japanese name akiyami, or autumn fever still persist in modern medicine. It was suggested that *Leptospira interrogans* serovar *icterohaemorrhagiae* was introduced to the western Europe in the 18th century by westward extension of *Rattus norvegicus* from Eurasia.⁽²⁾

The spirochetal aetiology, clinical features, pathology, immune response and immunotherapy of leptospires were studied independently in 1915 in Japan and Germany. In Japan, Inada and Ido detected both spirochetes and specific antibodies in the blood of Japanese miners with infectious jaundice. In Germany, groups of Unlenluth and Fromme studied German soldiers afflicted by 'French disease' in the trenches of northeast France. They detected spirochetes in the blood of guinea pigs which were inoculated with the blood of infected soldiers.⁽²⁾ Both the above groups had recognized

non-icteric and milder clinical form of illness. The spirochete they both described were forms of *L. interrogans* serovars *icterohaemorrhagiae* and *copenhagenie*, both of which are rat-borne.⁽¹⁾

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⁽¹⁾, while the potential for leptospiral disease in dogs was recognized, but clear distinction between canine leptospirosis with *L. interrogans* serovars *icterohaemorrhagiae* and *canicola* took several years. Leptospirosis in livestock was recognized some years later. During the 1920's to 1950's the milder forms of leptospirosis, the numerous related but distinct serotypes and the occupational relationship was made clear in Japan, Indonesia and Germany. The significance for domesticated animals other dogs was highlighted by work from the USA in the 1960's and 1970's, followed by the development of veterinary vaccines for serovar *pamona* at first, followed by others. In the 1970's and 1980's the ubiquitous distribution of *hardjo* infections in cattle and in humans involved with them were recognized. During this period the main advances made included redefinition of occupational risk, clarification of mode of transmission, mainly through genital carriers among animals and new method of diagnosis from blood like ELISA. Ideas about pathogenesis, virulence, immunity and vaccine protection were refined due to better understanding of the microbiology and immunological responses in infection by Leptospire. Antibiotic therapy with penicillin coupled with modern, general diagnostic and clinical management reduced the fatality rate.⁽¹⁾

Bacteriology :-

Taxonomy and classification :

The genus name *Leptospira*, meaning a very thin spiral was given by Noguchi in 1917.⁽⁵⁾

The family leptospiraceae was first described in 1979 and consisted of spiral shaped bacteria with hooked ends in the order spirochaetales. These organisms are the causative agents of leptospirosis, a zoonosis with a worldwide distribution. There are two taxonomic systems applied, they are serological classification and genotypic classification.

Serological classification :-

Before 1989, the genus *Leptospira* was divided into 2 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 the growth of the former at 13°C and growth in the presence of 8-azaguanine (225 µg/ml) and by the failure of *L. biflexa* to form spherical cells in 1M NaCl.

Both *L. interrogans* and *L. biflexa* are divided into numerous serovars defined by agglutination after cross absorption with homologous antigen. If more than 10% of the homologous titre remains in at least one of the two antisera on repeated testing, the two 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. Serovars that are antigenically related have traditionally been grouped into serogroups. While serogroups have no taxonomic standing, they have been proved useful for epidemiological understanding.

Genotypic classification :-

The phenotypic classification of leptospires has been replaced by a genotypic one, in which a number of genomospecies include all serovars of both *L. interrogans* and *L. biflexa*. Genetic heterogeneity was demonstrated some time ago, and DNA hybridization studies had led to the definition of 10 genomospecies of *Leptospira*. An additional genomospecies, *L. kirschneri*, was added later. Recently Centers for Disease Control (CDC), after an extensive study, have defined 16 genomospecies of *Leptospira*, adding five new genomospecies to those described previously, one of the five new was named *L. alexanderi*.⁽²⁾

The genomospecies of leptospira do not correspond to the previous two species i.e. *L. interrogans* and *L. biflexa*, but both pathogenic and non-pathogenic serovars occur within same species. Thus neither serogroup nor serovar reliably predicts the species of *Leptospira*.

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The reclassification of leptospires on genotypic grounds is taxonomically correct and provides a strong foundation for future classification. However, the molecular classification is problematic for the clinical microbiologists, because it is clearly incompatible with the system of serogroups which has served clinicians and epidemiologists well for many years.

Biology of Leptospires :-

The routine method of observation is darkfield microscopy of wet preparations. They are recognizable in clinical specimens such as blood, urine and cerebrospinal fluid (CSF) as spiral organisms. The leptospiral cell is about 6 - 20 μm in length, 0.1 - 0.3 μm in diameter. It comprises a peptidoglycan complex loosely referred to as cell wall arranged as a flexible hollow tubular right-handed helix, containing cytoplasmic and genetic material. The amplitude of the coil is about 0.10 - 0.15 μm and its wavelength is about 0.5 μm . They have numerous closely wound primary coils, so closely set together that they are difficult to demonstrate in stained preparations although they are quite obvious in the living state by darkfield microscopy. They are fairly rigid organisms with one or both ends hooked. As they move across the field, secondary coils appear and then disappear again, sometimes with a lashing motion. The movement of leptospires is characteristically vigorous, quite unlike the lazy undulating movement of other spirochetes. Leptospires exhibit two distinct forms of movement, translational and non-translational. The characteristic motility to leptospires results from the rotation of the flagellum, generating a helical wave that propels the helical cell in the opposite direction. The outer envelope acts as fluid mosaic. In fluid media they can travel about 7 - 10 μm in a second. In viscous fluid media the speed of movement is increased in proportion to the viscosity. Leptospires are attracted to more viscous environment by 'Viscotaxis'. In blood films or in semi-solid medium they show sinuous corkscrew like movement, occasionally bending and straightening again into the characteristically rigid form. This detailed description of the movement of leptospires is important because unless one is experienced in the work, it is easy to be misled by spiral artifacts that are normally present in blood films and which undulate as a result of Brownian movement.⁽⁸⁾

In leptospire, the organ for motility are a pair of flagellum, which are similar in structure and function to that of the other bacteria. At both ends of the helix of leptospire, a flagellum is subterminally inserted in the cell wall and penetrates to the outer aspect of the helix, where it runs through the interior of the coil in a groove to end at about the middle of the leptospire. The 2 flagella seldom overlap. The flagellar origin appears as a visible bulge below the surface. The whole is surrounded by a fluid membrane, the outer envelope (OE), separated from the cell by a periplasmic space. The membrane appears to have 3 or 5 layers. There is an unusually high lipid content in leptospire, located mainly in the cell wall or outer envelope.⁽⁷⁾

In some cultures leptospire appear in a 'granular' form. The granules are small optic structures, about 1.5 - 2.0 μm in diameter, inside which coiled remnants of the leptospiral all can be seen. Once thought to be a cystic stage of a life cycle, they are really spheroplasts. Formation of spheroplasts can be induced with ethanol, detergents, sodium deoxycholate, high salt conditions, lysozyme or mild heat. Some cultures tend to form spheroplasts spontaneously.⁽⁷⁾

Leptospire are obligate aerobes with an optimum growth temperature of 28 to 30°C. They produce both catalase and oxidase. 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 oxidation.⁽²⁾ They can desaturate fatty acids. Usually they do not require an unsaturated fatty acids in the medium and can use saturated fatty acids. Few strains are able to grow in a medium with acetate as the sole carbon and energy source.⁽⁹⁾

Culture Methods :-

Leptospire are grown routinely in liquid media, occasionally slightly thickened with liquid media demands the outmost care in their sterilization and quality control and in aseptic technique, to prevent contamination by extraneous air-borne or filterable water borne micro-organisms, including other leptospire.⁽⁷⁾

Liquid medium is used for growing leptospire intended for use as antigens in serological tests. It consists of a buffered salts base with or without peptone, to which either rabbit serum or other serum derivatives like bovine serum albumin or even the readymade enrichment supplement is added after filter sterilization.⁽⁸⁾

Semisolid media is prepared by adding 0.2-0.5% (w/v) agar to any suitable liquid medium. Agar appears to favour the multiplication of leptospire and semisolid medium is used when attempting the isolation of leptospire from blood or urine of patients or from animal samples or tissues. It is advantageous than the liquid media as it evaporates less rapidly and helps to maintain the virulence of freshly, isolated strains as subculturing is required less frequently. It is also used for preparing cultures for inoculation rabbits intravenously for production of hyperimmune serum. Fletcher's medium is one of the simple semisolid media; it consists of Agar and rabbit serum.⁽⁸⁾

Solid culture medium is used only for specialized purposes like separation of mixed cultures of leptospire, isolation, for detection of hemolysin production and for cloning. For best results the plates should be sealed to prevent evaporation and incubated at 28-32°C in a moist chamber or in a polythene bag. The colonies develop very slowly, so the plates are required to be incubated for at least 6 weeks before discarding them as negative.⁽⁸⁾⁽²⁾

Growth and Optimal Conditions :-

Leptospire grow by elongation of the cell body followed by constriction and separation at a flexible joint in the middle of the cell, creating two halves. A new flagellum develops at the new free end and grows in towards the middle of each daughter cell. Sometimes when separation fails very long forms of leptospire are formed sometimes in form of chains, particularly where the amounts of fatty acids on types of them are inadequate in the medium.⁽⁵⁾

Leptospire well adapted to their environmental conditions grow with a doubling time of 6-8 hrs in fluid or solid laboratory media at 28-30°C. Freshly isolated pathogens or environmental strains may take days or weeks to adapt to laboratory conditions, their doubling time may range from 14-18 hrs. The lag phase may last for days especially in new isolate, but is short for the laboratory cultures grown from large inocula subcultured from vigorously growing cultures. The stationary phase is reached at culture densities of approximately 10^8 cells/ml in serum media and 10^8 - 10^9 cells/ml or more in Tween albumin media. The culture density is enhanced if it is well aerated, and can stay at that level for days. The cultures stored in dark at room temperatures may be viable for years. The saprophytic strains grow faster.⁽⁵⁾

Leptospire are very sensitive to the environmental conditions, and if unfavorable they may take much longer time to grow.

The optimum temperature range for both the pathogenic and saprophytic leptospire is 28-32°C, but the extremes they can resist is from 11-42°C. Growth at 11-13°C is one of the differentiation criteria's between the saprophytic and pathogenic leptospire, as the saprophytic leptospire like *L. biflexa* show growth at these temperatures, whereas pathogenic leptospire do not.⁽⁷⁾

Leptospire die in acidic conditions at pH below 6.5 but can tolerate more alkaline conditions upto about pH 8.4. The optimum pH range at which the cultures grow well is pH 7.2 - 7.6.⁽⁷⁾

In case of salinity, most of the culture media for leptospire have molarity of salts about 0.05 M, which one third of that of other bacteriological media. Pathogens grow well at 0.85% NaCl at 37°C. Halophilic leptospire require sodium ion concentration of 0.22 - 0.44 M for growth and can tolerate concentrations upto 0.65 - 0.75 M.⁽⁷⁾

Leptospire die out rapidly in urine, sewage and badly polluted water, salt water has a deleterious effect on them. They are susceptible to heat treatment, i.e. they are killed within 10 minutes at 50°C or in 10 seconds at 60°C. They are rapidly killed by bile, trypsin, hypochlorite, detergents, and desiccation. They are sensitive to heavy metals, halogens, drying and reducing conditions. They can survive chilling to 4°C and freezing to -20°C in tissues like kidneys. In the laboratory they can be cryo preserved in liquid nitrogen and can also be lyophilized.⁽⁷⁾⁽⁸⁾

Distribution and Natural habitat :-

Leptospire have a worldwide distribution. These organisms may be free living or may live in association with human or animal hosts. The organisms are excreted in the urine of infected animals and may survive for days to months in freshwater, soil or mud. Pathogenic leptospire do not survive well in salt water or in environments with low pH or low humidity.⁽⁶⁾ Both pathogens and non-pathogens have been isolated from fast flowing rivers and still surface ponds. Soil and subsoil types and structure are important for survival in dry periods.⁽⁷⁾ The incidence of leptospirosis is much higher in warm-climate countries than in the temperate regions. This is due to longer survival of leptospire in damp soil with high humidity at ambient temperatures of 25 - 30°C, and at a slightly alkaline pH.⁽¹⁰⁾

Pathogenic leptospire gain entry into the hosts through the integument in moist conditions. In the host they circulate and grow in a aqueous milieu and may attach to renal tubular cells in carrier animals, passing in urine into surface waters where they feed by attaching to surfaces where long-chain fatty acids are available. Pathogenic leptospire adapt to environmental conditions including the salinity and temperatures of febrile animals, renal tubular and bladder urine and soil and surface water.⁽⁷⁾ Humans are dead end hosts in the chain of transmission, with possible incidents of person-to-person transmission rarely being reported.⁽⁶⁾

The distribution of *Leptospira* serovars varies by region and host species. Some commonly recognized associations between serovars and their hosts include canicola with dogs, pomona with cattle or swine, ballum with mice, and icterohaemorrhagiae with rats. A knowledge of the predominant serovars in the geographic region of interest for the development of effective methods for the diagnosis of leptospirosis.^{(6) (45)}

DISEASE

General Introduction :-

Leptospirosis has been described as a 'zoonosis of protean manifestations'.⁽¹⁰⁾ General and universally applicable principles govern the mode of infection, mechanisms of production of symptoms and signs, clinical features, potential outcomes, mechanisms of immunity, transmission and management.⁽¹¹⁾

Severe forms, caused by serovars like australis, autumnalis, batavia, copenhageni, icterohaemorrhagiae, javanica or lia, are frequently fatal if untreated; their main features are jaundice, haemorrhage, potentially fatal kidney and liver failure, meningitis and a myraid of other symptoms some patients may have very mild anicteric and non-haemorrhagic symptoms of an infection by a serovar that can otherwise cause a severe infection. Mild forms of leptospirosis range from a febrile incapacitating illness lasting 10-20 days, comprising severe muscle pains, meningism and mild renal incapacity, to an imperceptible subclinical infection. Examples of serovars that usually cause a mild type of leptospirosis are ballum, hardjo, grippityphoso, pomona and tarassovi.⁽¹¹⁾

Pathogenesis :-

The aetiological agents of leptospirosis are pathogenic leptospires, which are small thin motile helical bacteria. Their primary source is the surface of the renal tubules in the kidney of an excreting carrier animals pass urine containing leptospires into the surroundings,

contaminating soil, groundwater, or mud. Leptospire survive in moist environments at temperatures from about freezing to about 40°C. They are aquatic bacteria at home in wet or moist environment, they die when they dry under normal environmental conditions.⁽¹¹⁾ (40)

Portals of Entry :-

Leptospire enter fresh host from urine, water, soil or mud by penetrating small sometimes invisible abrasions in skin or body surfaces, possibly also by inhalations, even through conjunctival sac, the penetration and invasion of tissues is presumably accomplished through a barrowing motion produced by a pair of axial filaments and release of hyaluronidase.⁽⁵⁾⁽¹¹⁾ Drinking or inhalation of contaminated water can also result in leptospirosis. The maternal blood can also infect the fetus.⁽¹¹⁾

Entry into the Host :-

A very small number of leptospire i.e. 1 to 10 cells can cause a fatal infection in a susceptible animal. Once entered leptospire spread rapidly; they can be found in the blood stream minutes after subcutaneous or intramuscular injection and almost immediately after intraperitoneal injection. Motility plays an important role in their spread as they are highly motile in a viscous environment like that of body fluids as compared to watery fluids.⁽¹¹⁾

Survival after Entry :-

Virulent leptospires are not affected by the IgM antibodies and its complement components acting alongwith dysozymes, and they survive and grow in the body.⁽¹¹⁾

The body environment is very different from the wet soil or water or urine from which the invading leptospires have come. There is a large increase in salt concentration and osmotic pressure. Virulent pathogenic leptospires can survive at 37°C where avirulent leptospires and non pathogens die.⁽¹¹⁾

A latent period corresponding to the incubation period in clinical leptospires occur after infection or experimental inoculation, during which leptospires multiply in the body without causing *major subjective* or objective ill effects. The growth rate in the body corresponds to a doubling time of 6-8 hrs. A 10 fold increase in numbers thus takes about a day.⁽¹¹⁾

Leptospirosis in Animals :-

Leptospirosis occurs in all farm animals species and is an important zoonosis. It causes septicaemia, interstitial nephritis, haemolytic anaemia and abortion in most species and may be the cause of equine periodic ophthalmia.(12)

Epidemiology :-

L. pomona is the commonest infection in all animals. Its survival in the environment depends largely upon variations in soil and water conditions in the contaminated area, it is particularly susceptible to drying and to changes in pH. A pH lower than 6 and higher than 34-38°C are detrimental. Moisture is the most important factor governing the persistence of organism in bedding or soil, it can persist for as long as 183 days in water saturated soil but only for 30 minutes when soil is air dried. The organism survives in free, surface water for very long periods, the survival period is longer in stagnant than in flowing water.(12) (45)

L. pomona infection is recorded in cattle, pigs and horses, and the ovine infection has been provisionally identified as *L. pomona*, and the disease has been produced experimentally in these species by injection of bovine and porcine strains of the organism. Because of the severity of the systemic disease, the high rate of abortions and abnormalities of milk, its importance is greater even than its prevalence. The disease has been experimentally produced in cattle. *L. canicola* infection has been recorded in cattle and in pigs and antibodies to it have been detected in horses. *L. icterohaemorrhagiae* is a rare isolation in large animals but have been reported in cattle and pigs, and serological evidence of infection has been found in the horse. *L. grippotyphosa* from cattle and goats, and positive serological tests

have been obtained in horses. *L. sejroe*, *L. hebdomadis* and *L. australis* infection have been observed in cattle.⁽¹²⁾

Calves and lambs are highly susceptible and are likely to develop the septicaemic form disease. Strong immunity after attack may occur.⁽¹²⁾

The source of infection is usually an infected animal which contaminates pasture, drinking water and feed by infected urine, aborted fetuses and infected uterine discharges. The semen of an infected bull may carry leptospires and transmission from such a bull to heifers by coitus and artificial insemination has been observed. For most leptospiral serovars, the most important carrier hosts are rodents but *L. pomona*, *L. tavarossi* and *L. bardjo* are adapted to agricultural animals as carrier hosts, with obvious epidemiological implications.⁽¹²⁾

(40)

Different rodent species may be reservoirs of distinct serovars, but rats are generally carriers or maintenance hosts for serovars of the serogroups icterohaemorrhagias and Ballum.⁽¹²⁾

Because of rapidly accumulating evidence of a high rate of infection with leptospira in wildlife these animals are suspected of playing a significant role in the spread of disease to domestic animals. Entrance of the organisms into the body occurs most probably through cutaneous or mucosal abrasions. Transplacental transmission is not common but neonatal infection, probably contracted in utero, has been reported. Oral dosing is an unsatisfactory method for experimental transmission as compared to infection and installation into the nasal cavities, conjunctival sac and vagina. Because of the importance of water as a means of spreading infection new cases are most likely to occur in wet seasons and low lying areas, specially when contamination and susceptibility are high.⁽¹²⁾

Urine is the chief source of contamination because animals, particularly pigs even after clinical recovery, may pass leptospires in the urine for long periods sometimes upto 2 months cattle have been shown to have leptospiuria for a mean period of 36 days with highest excretion rate in the first half of this period. Horses have leptospiuria for about 4 months though is slight degree. In goats leptospiuria persists for atleast one month. The leptospires may persist in the kidney for much longer period than they can be recovered from the urine by routine laboratory methods. Urine drinking is a common practice in some dairyherds and can be a means of spreading leptospirosis actively.(12) (45)

The disease appears to be worldwide in its distribution. In general terms the disease is most common in certain areas or seasons when the climate is warm and humid, soils are alkaline and there is an abundance of surface water.(12)

After penetration of the skin or mucosa the organisms multiply rapidly in the liver and then migrates to, and can be isolated from peripheral blood for several days until the fever subsides, at which time antibodies appear in the blood stream and organisms in the urine. Persistence of leptospires in renal lesions lead to prolonged leptospiuria. Much of the pathogenesis of leptospirosis derives from the damage to capillaries which is common to all serotypes. Some serotypes also produce endotoxins, but some also produce haemolysis, so that severe haemolysis may add anaemic anorexia and even haemoglobinuric nephrosis to the basic vascular lesion. In the acute phase of the disease the affected animal may therefore die of septicemia or haemolytic anaemia or a combination of the two. Subsequently death may be due to uraemia or hepatic insufficiency. Renal lesions develop due to the persistence of infection over there. Interstitial nephritis and uraemia may then follow. The other common

sequel is abortion which is caused by the death of the fetus, with or without placental degeneration, both effects resulting from invasion of the conceptus during the septicaemic stage of the disease. Abortion always occurs several weeks after the septicaemia because of the time required to produce the changes in the fetus, which is usually degenerated at birth, and has obviously been dead for more than 24 hours. Abortion occurs only in the second half of pregnancy, probably due to the greater ease of invasion of the placenta at this stage.⁽¹²⁾
(41)

Cattle :- Although the mortality rate is low (5%) in this species, the morbidity rate is usually high as determined clinically and serologically and may approach 100% of in contact animals. In calves the mortality rate is much higher than in adult cattle. A high rate of abortion (upto 30%), and loss of milk production are the major causes of loss but death in calves may also be significant.⁽¹²⁾ (43) (45)

Pigs :- In infected herds the incidence of positive reactors to serological tests is high, and in large affected pig populations averages about 20%. Economic losses are about equally divided between abortions and deaths of weak and susceptible new born pigs.⁽¹²⁾ (44) (45)

Sheep and Goats :- The disease in these species has been reported from many countries. Death of animals and loss of condition in mildly affected animals are the main causes of loss. Morbidity rate of 100% is common in sheep and that of goats is 45%.⁽¹²⁾ (41) (45)

Horse :- The disease is relatively mild in horses, and losses except for blindness due to associated periodic ophthalmic are negligible. When groups of horses are known to be infected, an average of upto 30% of the adult horses give positive serological results.⁽¹²⁾ (45)

Dogs :- Serovars *canicola* and *icterohaemorrhagias* are important causes of canine leptospirosis throughout the world. Acute leptospirosis, commencing with vomiting, fever and red eyes, progresses to nephritis (arched back, immobility, blood stained urine and faeces), dehydration resulting into death within 4 days. Rapid collapse and death within hours characterizes a 'peracute' form of *icterohaemorrhagiae* infection. Dogs that survive are often renal excretors and a risk to humans close to them.⁽¹⁾ (42) (45) (46)

Rodents and Marsupials :- Guinea pigs and hamsters, and some marsupials, are extremely susceptible to infection with leptospire of several serovars. Rats and mice, on the other hand, are notoriously common and important carriers and excretors of serovars causing severe forms of leptospirosis in humans and animals. In guinea pigs and hamsters the signs are ruffled fur, immobility, red eyes and fever commencing about 1-3 days after inoculation. Bloody diarrhoea, red urine, jaundice and convulsions may occur, corresponding to haemorrhages and nephritis. Death occurs in 3-7 days, the picture is same for feral rodents and marsupials.⁽¹⁾ (45)

Birds :- Chick embryos can be infected by chorioallantoic (CA) or amniotic or yolk sac inoculation of 9-12 day old eggs. Following CA inoculation, haemorrhages can be seen on the CA membrane in 3-7 days. The embryo and membranes develop haemorrhages and jaundice and the embryo may die. If it does not, it may hatch joundiced and leptospire may circulates in the blood.⁽¹⁾

Poikilothermic animals :- Leptospires have been isolated from the kidneys of frogs, and fish were experimentally infected without evidence of illness. Leptospires of serovar *bim*, a common cause of leptospirosis in Barbados, were isolated there from toads.⁽¹⁾

One of the important features of leptospirosis is its transmissibility to human and it represents an occupational hazard to butchers, farmers and veterinarians. Human infection is most likely to occur by contamination with infected urine or uterine contents. Although leptospires may be present in the milk for a few days at the peak of fever in an acute case, the bacteria does not survive for long time in the milk and does not withstand pasteurization.

Leptospirosis in Humans :-

The clinical presentation of leptospirosis is biphasic, the acute, or septicaemic phase which lasts for about a week, it is followed by the immune phase, characterized by antibody production and excretion of leptospire in the urine. Most of the complications of leptospirosis are associated with localization of leptospire within the tissues during the immune phase, and thus occurs during the second week of infection.(10)

The great majority of infections are either subclinical or of a very mild severity and will probably not be brought to medical attention. The majority of recognized cases are present with a febrile illness of sudden onset, of which the symptoms include chills, headache, myalgia, abdominal pain, conjunctival suffusion and less often a skin rash.(10)

Pathology and Pathogenesis :-

The mechanisms involved in the pathogenesis of tissue injury are -

- a) Direct bacterial invasion
- b) Non specific factors
- c) Immunologic reactions

After gaining entry, the organisms spread through the blood stream to all organs and multiply. Leptospire at this stage can be recovered from blood and all tissues (Leptospiremic phase). Leptospire cause extensive endothelial injury resulting in hemorrhages in the mucous membranes, skin, lungs, kidneys, liver and other organs. Liver and renal damage occurs mainly due to direct bacterial invasion. Transudation of fluid from the vascular compartment

causes hypovolemia. Hypovolemia is common and attributed to decreased fluid intake, increased insensible fluid loss and increased capillary permeability. The combination of hypovolemia, hyperviscosity, intravascular coagulation alongwith cytokines and complement activation lead to various systemic manifestations of Leptospirosis.(4)

Leptospiral infection produces IgM antibodies early (4 -7 days) followed by IgG. The agglutinating antibodies opsonise the leptospires and the reticulo-endothelial system effectively clears the organisms from the blood stream. Cell mediated immunity probably plays a role in eliminating leptospires from the tissues. Meningeal inflammation and uveitis occurs due to immunological reactions.(4)

Clinical Manifestations :-

Leptospirosis is an acute febrile systemic disease whose manifestations result from the effects of a general vasculitis. Its clinical features range from a mild, anicteric illness to a severe life threatening disease with jaundice, renal failure or bleeding.

The incubation period ranges between 2-30 days, usually 5-14 days. Leptospirosis is biphasic illness. The first or 'septicemic' phase lasts 4-7 days and is characterized by sudden onset of headache, severe myalgia and fever. Abdominal pain and vomiting and conjunctival suffusion may follow. The patient may then be in febrile stage for a few days after which the second or immune phase begins, associated with fever, meningeal signs, bleeding and other systemic involvement.(4)

Anicteric Leptospirosis :-

A smaller proportion of infections, but the overwhelming majority of the recognized cases are present with febrile illness of sudden onset. Alongwith the above mentioned symptoms except jaundice the symptoms found here are chills and skin rash. Skin rash if present, it is transient, lasting less than 24 hrs. This anicteric syndrome usually lasts for a week and its resolution coincides with the appearance of antibodies. The fever may be biphasic and may recur after a remission of 3 to 4 days. Headache, often severe, resembles to dengue with retro orbital pain and photophobia. Myalgia affecting lower back, thigh and calves is often intense. Aseptic meningitis may be found in around 25% of all leptospirosis cases. Mortality is almost nil in anicteric leptospirosis, but death from massive pulmonary hemorrhage can occur. The differential diagnosis must include common viral infections, such as influenza, human immunodeficiency virus seroconversion, and in the tropics, dengue, in addition to the bacterial causes of fever of unknown origin, such as typhoid.⁽²⁾

Icteric Leptospirosis :-

Icteric leptospirosis is a much more severe disease in which the clinical course is often very rapidly progressive. Severe cases often present late in the course of the disease, and this contributes to the high mortality rate which ranges between 5 to 15%. Between 5 and 10% of all patients have icteric form of disease. Jaundice in leptospirosis is not associated with hepatocellular necrosis, and liver function returns to normal after recovery. Serum bilirubin requires many weeks to come to the normal level. Moderate rise in transaminase and alkaline phosphatase levels is usual. Leptospirosis is a common cause of acute renal failure (ARF) which occurs in 16 to 40% of cases. Serum amylase levels are often raised significantly in association with

ARF. Thrombocytopenia occurs, and is a significant predictor for the development of ARF.(2)

The occurrence of pulmonary symptoms in cases of leptospirosis has been reported. The severity of respiratory disease is unrelated to the presence of jaundice. Patients may present with a spectrum of symptoms, ranging from cough, dyspnea, and hemoptysis to adult respiratory distress syndrome. Intra-alveolar hemorrhage has been reported. Pulmonary hemorrhage may be severe enough to cause death.(2)

Cardiac involvement in leptospirosis is common but may be underestimated. Fatal myocarditis has been described. Clinical evidence of myocardial involvement, including abnormal T waves, was detected in severe icteric cases. A mortality rate of 54% has been reported in severe leptospirosis cases with myocarditis. Repolarization abnormalities on ECG are as a poor prognostic indicator in severe leptospirosis cases.(2)

Ocular involvement :-

Conjunctival suffusion is seen in the majority of patients. Conjunctival suffusion in the presence of scleral icterus is said to be pathognomonic of Weil's disease. Anterior uveitis, either unilateral or bilateral occurs after recovery from the acute illness in a minority of cases. Uveitis may be present weeks, months or occasionally years after the acute stage. In most cases uveitis is presumed to be an immune phenomenon, but leptospire have been isolated from human and equine eyes. Late onset of uveitis may result from an autoimmune reaction to subsequent exposure.(2)

Other Complications :-

Acute infection in pregnancy has been reported to cause abortion and fetal death. Leptospire have been isolated from human breast milk. Rare complications include cerebrovascular accidents, rhabdomyolysis, thrombotic thrombocytopenic purpura, acute acalculous cholecystitis, erythema nodosum, aortic stenosis, Kawasaki syndrome, reactive arthritis, epididymitis, nerve palsy, male hypogonadism, and Guillain-Barre syndrome, Cerebral arthritis resembling Moyamoya disease.⁽²⁾

Economic Importance of Leptospirosis :-

The main economic importance of leptospira lies in their ability to produce infection in animals and humans. The economic factors in leptospirosis include direct or indirect costs of the following -

Abortion, failure to thrive, loss of milk production, and associated veterinary costs in domestic and commercial livestock and dogs, with potential for malnutrition and impoverishment in individuals and communities dependent upon animal sources of protein, especially in subsistence economies.

Vaccination and surveillance (vaccines, veterinary attention, laboratory tests, evaluation and administration) including testing and certification of serum samples from large numbers of livestock before international transport.

Human illness, suffering, medical care, and loss of productive working time and capacity.

Production and evaluation of vaccines and vaccination programs for animal and human.

Rodent control in livestock management, pastures (including sugar cane plantations and rice fields), and food processing.

Occupational disease surveillance and control, protective clothing, and insurance for workers in industries where there is a significant risk (e.g. milkers of cows, slaughterers, assistants, inspectors, and laborers at abattoirs, military personnel's).(5)

**REVIEW
OF
LITERATURE**

REVIEW OF LITERATURE

Leptospirosis was first defined as an independent class of disease in the second half of the 19th century. The separation of Leptospiral jaundice from other infectious diseases of liver occurred in two stages, the first was clinical and the second, nearly thirty years later, bacteriological. Human illness, characterized by fever, jaundice, renal involvement and nervous symptoms was established as a separate clinical entity by Prof. Adolf Weil in 1886 from a study of four cases. The infective agent was not known at that time. The term 'Weil's Disease' was first used by Glodschmidt in 1887 after studying two cases which presented the same features like febrile illness and severe nervous symptoms, jaundice, signs of renal involvement, and enlargement of liver and spleen were the other effects. Recurrence of fever for five to six days, following a febrile period of one to seven days, occurred frequently. In 1892 Jager reported similar illness among soldiers and explained it to be due to bathing in the river. In 1908, Hunter laid emphasis on the severity of muscular pains and nephritis. Enlargement of the spleen was said to be usual. Among the etiological features, he noted that the incidence was highest among boys and young men and around 90% of the patients were male. Stimson in 1907 gave the name *Spirochaeta interrogans* after performing the silver impregnation staining of the renal tubules of a patient who had died of jaundice during an outbreak of yellow fever. In 1914, Wolbach and Binger isolated leptospire from water pond by filtration, which they named as *Spirochaeta biflexa*. In 1916, Inada *et al.* discovered the causative agent and named it *Spirochaeta icterohaemorrhagiae*.⁽²⁷⁾

In 1918 the name 'Leptospira' (thin spirals) was coined by Noguchi after a detailed microscopic and cultural observation, still believing it to be the causative organism for yellow fever.

Microscopically and culturally identical but antigenically different leptospires were at first regarded and named as separate species, but later included as serotypes or serovars of a single genus, *L interrogans*. Till 1930's many of the important serovars prevalent throughout the world, and their hosts, sources, were discovered. In the next 2 decades most of the research was related to the discovery of new serovars, some responsible for milder anicteric types of leptospirosis. There was little progress and much frustration in attempts to understand nutrition or the composition of antigenic fractions compared with the knowledge of the other bacteria. Electron microscopy revealed much of the detail of structure during the 1960's and 1970's.⁽⁷⁾

In USA, a widespread leptospirosis in cattle and pigs, due to *L pomona*, stimulated significant research into nutrition and cultivation of leptospires, much of it emanating from the laboratory of J B Wilson in Wisconsin during the 1980's, and continued by his former students thereafter. The most important single advance was probably the discovery that the only carbon and energy sources were the long-chain fatty acids essential for growth. They were toxic and had to be presented in a non-toxic form, detoxified by serum or serum albumin or another detoxicant in the medium. Bovine albumin oleic acid culture media improved yields upto 100 times those in rabbit serum media, especially on aeration, and also allowed the isolation of some strains unable to grow in rabbit serum media.⁽⁷⁾

The profusion of isolates of many previously unknown serovars of leptospires necessitated correct identification for diagnosis, prognosis an epidemiology, and for the development of vaccines. Consequently, leptospirosis researchers developed a preoccupation with serological classification, based only on absorption and cross-agglutination of antiserum (Abdussalam *et al.* 1972). 'Factor analysis'

(Kmety 1967) using batteries of absorbed sera to simplify identification was seldom used. ELISA methods were developed to analyze non-agglutinating as well as agglutinating antigens (Adler *et al.* 1980) and applied with monoclonal antibodies to identify epitopes involved in immunity, or useful for classification (Adler and Faine 1983). The current method of genetic classification was adopted in 1994 (Ellis 1995).⁽⁷⁾

Much of the work on leptospires and leptospirosis has been supported or stimulated not by physicians or microbiologists but by the veterinary public health sources and veterinarians because the economic consequences of leptospirosis are loss of animal productivity and human food supplies, as well zoonotic risks to humans.⁽⁷⁾

In animals, leptospirosis has been reported from almost all the mammals, and leptospires has been isolated from almost all of them. The prevalence is almost worldwide.⁽²⁸⁾

In India, research on leptospirosis in veterinary medicine was undertaken in an organized manner during the post-independence era. The relationship between leptospires and host is easily 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 organisms in urine, upto 100 million leptospires/ml. These animals are a potent source of infection, responsible for maintaining disease cycle in nature. Domestic and farm yard livestock may also become infected either as maintenance hosts of leptospires, specific for their own host species or may become accidental hosts to leptospires from other species. Wild life is the natural reservoir for many pathogenic leptospires, particularly the rodent family. Rat (*Rattus norvegicus*) is the predominant natural

carrier of leptospires chiefly *icterohaemorrhagiae* in most regions of the world. In India, it is *Rattus rattus*, which serves as a reservoir for several serovars.(28)

In India, the relationship between the carrier and the causative species was established in 1939, when Das Gupta BM was successful to isolate *icterohaemorrhagiae* from rats and Bandicoots in Andamans followed by Lahiri M N in 1941. Isolation of *L. hebdomadis* and *L. pomona* from Goats in 1961 and 1960 was done by Pande, P.G. *et al.* Establishment of the relationship between Dogs and *L. canicola* was done in 1966.(28) Alex J.C. *et al.* isolated *L. autumnalis* from a Bandicoots and a rat and identified it as a infecting species in one of the human samples in Madras in 1993.(29)

In 1999, Zamora, J and Riedemann, S. isolated *Leptospira spp.* from 36 rodents kidney tissue samples and cultured it in EMJH medium.(30)

In 2000, N.L. Gangadhar *et al.* in an attempt to isolate leptospira from kidneys of *Rattus rattus*, *Bandicota bengalensis* and *Bandicota indica* in Bangalore, isolated 296 spirochetes from 1,348 kidneys. Out of which from a batch of 56 isolates 23 isolates were identified as *L. inadai*. It was the first record of isolation of *L. inadai* from rodents.(31)

CURRENT STATUS

CURRENT STATUS OF LEPTOSPIRAL INFECTIONS

Leptospirosis is now considered as one of the emerging infectious disease with its prevalence all over the world. Though it is primarily viewed as a disease of animals, but the large number of human cases occurring every year all over the world has made everybody to realize the importance destructive capabilities of this infection. It has a wide variety of animal hosts and most of the animal carriers can spread the infection to man. In most parts of the world, leptospirosis is primarily an occupational disease and agricultural manual workers account for more leptospirosis cases than any other occupational group. The interaction of man with animals and environment occurs in various other spheres of life and spread of infection from animals and environment can take place in many such instances. Because of all these, leptospirosis is one or the other form has been reported from virtually every part of the world.⁽¹³⁾

Situation of leptospirosis worldwide:

Leptospira icterohaemorrhagiae was discovered first by the Inada and Ido in 1915 in Japan, but the disease caused by it i.e. Weil's disease was known since 1886. After that many different names were given to the disease depending upon occupational groups involved and the nature of the disease, few examples of which are cane cutter's disease in Australia, rice field leptospirosis in Indonesia and fort brag fever in the US. Weil's disease, which is one of the severe forms of the disease, occurs in many countries including India and other Southeast Asian countries, China, Continental Europe and England. Leptospirosis exists in all the five inhabited continents and in

a large number of countries. It occurs in tropics, subtropics and in temperate zones.(13)

Few of the recent reported outbreaks of leptospirosis in various countries are given in the following table.

Country	Number of cases	Period
Azores Islands	389	1992-2000(14)
Brazil	28,360	Till 1999(15)
China	500,000	Till 1999(15)
India	2,000	Till 1999(15)
Hawaii	52	1998-1999(16)
Sri Lanka	1500	Jan to Oct 2003(18)

Other than the above given data, it is estimated that there are around 100 to 200 identified cases annually in US.(15) Leptospirosis has been a cause for concern as it has been reported from about 32 countries all over the world with substantial number of infections.(17)

Situation of Leptospirosis in India :-

Leptospirosis was first reported in India by Taylor and Goyal in 1929 when they observed 64 cases and succeeded in isolating the leptospire from 24 of them. Reports of leptospirosis were very scanty till 1980's after which they became frequent. According to the currently available data, occurrence of leptospirosis is very common Tamil Nadu, Kerala and Andaman Islands. Recent outbreaks have been reported from various states including Karnataka, Gujarat, Bihar and Maharashtra where it has been very frequent. Serological

evidence of leptospiral infections has been reported from West Bengal and Uttar Pradesh.⁽¹³⁾

The scenario of leptospirosis in India is as follows -

Place	Suspected cases	Period
Mysore	64	1997(13)
Surat	281	1937(13)
North Andaman	83	1997(13)
Pondicherry	250	1998(13)
Chennai	731	1998(13)
Cochin	227	1998(13)
Bangalore	44	1998(13)
Bihar	22	1998(13)
Manipal	44	1998(13)
Assam	127	1998(13)
Andaman	60	1998(13)
Valsad	448	1997(19)
Orissa	56	1999(20)
Surathkal (Karnataka)	15	1999(21)
Delhi	32	2000-01(22)

Alongwith the above data, 1679 cases of leptospirosis have been reported from various parts of India, by the WHO in one of the Weekly Epidemiological Records of 1999.⁽¹⁷⁾

Situation of Leptospirosis in Maharashtra and the study area: -

In Maharashtra leptospirosis has been very often reported from the places like Nagpur, Pune, Mumbai etc. There was a suspected outbreak in Nagpur in 1997 with number of the suspected cases going to 20. In 1998, 128 suspected cases were reported from Pune, out of which 42% were confirmed by MAT.⁽¹³⁾

In Mumbai, Lahiri was the first to identify and isolate leptospirae from a human case, on inoculation of a guinea pig.⁽¹⁹⁾ He was followed by P.M. Dalal who reported five cases of Leptospirosis in 1960.⁽¹³⁾ Then there were reports of the disease in 1956 and 1967.⁽¹⁹⁾

Then after a big interval the disease again emerged as a major problem in 1998-1999 when there were 111 reported cases with 3 casualties. And in year 2000 the casualties raised upto 36 in Mumbai and Thane District, which created a panic.⁽²³⁾

In 2000 leptospirosis was first detected in far-flung suburbs like Vasai, Bhayander and Mira Road and later in the close suburbs. The total number of suspected cases was around 290 till August 2000.⁽²⁴⁾ In year 2002, the infection had claimed 27 lives and around 106 patients were admitted to various hospitals for suspected leptospirosis.⁽²⁵⁾ The latest report of leptospirosis in Mumbai region was given by Sushil Shah and Nilesh Shah in April 2003; they found 516 patients having IgM antibodies for leptospira.⁽²⁶⁾

From all the current scenario of leptospirosis, it has become a major cause of concern to all. This was the reason why we undertook the following work so as to help us to know more about this disease. And we targeted the rodents because it was one of the root source and reservoir for the leptospiral infections. This work was

necessary to help us to tackle this problem in the transmission phase itself, and to help us to predict probabilities of the future outbreaks in the study area.

**AIMS
AND
OBJECTIVES**

AIMS AND OBJECTIVES

- ✓ (1) Collection of rodents from the Mumbai City and its suburbs.
- ✓ (2) To study the prevalence of various rodent species in the study area.
- (3) Processing of samples of ~~dead~~ rats for isolation of Leptospira.
- ✓ (4) Collection of Blood and Urine samples from the rodents.
- ✓ (5) Study the blood and urine samples of the rodents using the following methods
 - ✓ (A) Microscopy -
 - I) Dark Field Microscopy (DFM).
 - II) Staining techniques.
 - ✓ (B) Serological Tests -
 - I) Slide Agglutination test (SAT).
 - II) Microscopic Agglutination Test (MAT).
 - ✓ (C) Blood and urine culture in selective media for leptospira.
- ✓ (6) Culture isolation and confirmation of the results with respect to the standards.

Real Time
PCR
P. ^{W31} _{in} L V-1
g
PCR L

**MATERIALS
AND
METHODS**



1. Collection of rodents from the Mumbai City and its suburbs :

As stated in the introduction, Leptospirosis is one of most widespread zoonotic disease with a worldwide prevalence. Leptospirosis has a very wide host range, which includes almost all the mammals, few amphibians, etc. It originates from the animals, among which the rodents act as the primary reservoirs, because once infected the rodents acts as carriers for their lifetime, shedding leptospires through their urine, which in turn infects other animals and humans.

Leptospirosis has become a major cause of concern for the human population of Mumbai city, as there have been many outbreaks in the recent past, which resulted into a severe loss to the community. Rodents have been of equal importance due to the threat they pose to the community. Rodents have been the vectors for very severe diseases like Plague, Rat bite fever, Leptospirosis etc. They have also cause loss economically due to the loss to the food grains they cause. Rodents are present almost in every part of the study area.

The study area i.e. the Mumbai city and its suburbs are covered by the Mumbai Municipal Corporation (MMC). For the purpose of proper administration the whole area is divided into 24 wards which are as follows:-

Wards	Area
A	Colaba
B	Sandhurst Road
C	Marine Lines
D	Nana Chowk
E	Byculla
F/s	Parel
F/n	Matunga

G/s	Elphinston
G/n	Dadar Plaza
H/e	Bandra (East)
H/w	Bandra (West)
K/e	Andheri (East)
K/w	Andheri (West)
L	Kurla
M/e	Chembur (East)
M/w	Chembur (West)
N	Ghatkopar
P/s	Goregaon
P/n	Malad
R/s	Kandivali
R/n	Dahisar
R/c	Borivali
S	Bhandup
T	Mulund

All the above 24 wards have one Pest Control Officers (PCO's) who look after the health related problems in their respective wards. These PCO's work under the supervision of the Insecticide Officer of the MMC.

The Municipal Rat Destruction Establishment (MRDE), and unit of MMC was established around 1937, in the Haffkine Institute campus, to undertake work of routine plague surveillance from the rodents which were collected from the various wards of the Mumbai city. It has played a major role during few of the suspected plague outbreaks, which took place in various parts of India. The plague surveillance from the rodents is carried out as a routine activity in MRDE. MRDE receives around 1500 dead rodents daily from various wards. These rodents are either trapped live or killed by the rat

catchers of different wards, which after killing are transported to MRDE, where they are checked for plague surveillance and then discarded.

For the present work the rodents were obtained from MRDE. The collection of rodents was distributed into two parts. The first part was concerned with the diagnosis of leptospirosis from the dead rodents. For this the rodents, which came to MRDE for routine plague surveillance work, were used. These rodents were first killed by the MMC staff and then brought to the MRDE unit. These rodents were assorted according to their respective wards. While selecting the rodents for the leptospiral investigations, the freshly killed rodents were preferred. For the next part of the work which required the live rodents, the PCO's of the desired wards were informed. These PCO's then placed traps at different locations in their respective wards and when the rodents were trapped they were transported to the MRDE unit, from where we brought them to our lab for sample collection.

For trapping and maintaining the live rodents in the lab, the traps and the cages were provided by the MMC and the Dept. of Zoonosis respectively. The traps and the cages used for the above purpose were as follows -

Wonder Traps -

These were the traps, which were of two sizes the bigger one to trap rodents like *Bandicota spp* and other bigger rodents. The other type which was of medium size were preferred to trap the small sized rodents like *Rattus norvegicus* and *Rattus rattus*. These traps were available commercially.

Cages -

The use of cage was applied when the rodents were to be maintained in the lab for few days. The cages which were used are called 'D' cages. The name 'D' is given due to its structure. These cages were designed by the Haffkine Institute itself. There was a facility to keep water and food vessels in the cages for the rodents.



2. To study the prevalence of various rodent species in the study area :

To know the status of the species wise distribution of the various rodent species in the study area, a survey was undertaken with the help of MRDE. This survey included the screening of the areas, which were considered to be highly populated or endemic to certain species of the rodents. Then a list of commonly occurring rodent species in the study area was drawn which is as follows -

For the *Rattus* genus the species commonly found were -

Rattus rattus and,

Rattus norvegicus

For the *Bandicota* genus they were -

Bandicota bengalensis and

Bandicota indica

And other species which were commonly found were -

Mus musculus and

Suncus cereleus

All these rodents were distinct and possessed characteristic morphology. The morphological characters and the distinguishing features of the above rodents were as follows -

I. *Rattus rattus* :- (Common house rat)

1) Normally found near the human dwelling places. It is cosmopolitan, occurring practically all over India in both wild and commensal forms. Burrows in floors and roofs, mostly arboreal.

2) It is medium sized rat with the weight ranging between 200 to 280 gms. The body is slender shaped, elongated. Snout is narrow, long, pointed and fleshy. Eyes well developed and large.

3) Ears well developed, large enough to cover the eyes when folded.

4) Total tail length is longer than total length of head and body together. Rings are clear on tail and range between 260 to 290.

5) Fur is smooth and bicoloured i.e. darker and shiny black on back and higher on the belly.

6) Females have 5 pairs of mammary glands.

II. *Rattus norvegicus* :- (Norway rat or Brown Rat)

1) Comes under peridomestic form of rats, as it prefers living away from human dwellings.

2) It has complicated and net like burrows, mostly found in sewer drains, stables and open grounds near residential areas.

3) Body is cylindrical, elongated with large and sharp snout and blunt muzzle, body weight ranges between 300 to 450 gms.

4) Eyes and ears shorter as compared to *R. rattus*, ears densely covered with hair.

5) Length of tail is shorter than total length of body and head. The tail does not possess rings but has matt scales.

6) Fur is coarse, thick and bicoloured, deep brown on the dorsal side and light brown on head and abdominal regions.

7) Females have 6 pairs of mammary glands.

8) This species is known to be great fighters.

III. *Bandicota bengalensis* :-

1) This species is a serious pest of agriculture and stored grains and has an important role in transmission of diseases to human and its live stock.

2) Likes to live alone in mixed type multi channeled burrows, both commensal and wild in habitat.

3) Eyes and ears are short as compared to its body.

4) Tail length shorter than the total length of the head and body together. Rings though not clear are uniform ranging between 200 to 250.

5) Females have 8 to 9 pairs of mammary glands.

6) Fur is spinous, black and coarse.

7) Body weight ranges between 400 go 500 gms.

IV. *Bandicota indica* :-

1) Largest rat of the Bandicota genus. Peridomestic and wild in habitat. Burrows deep and erects boat shaped structures.

2) Shape similar to *Rattus norvegicus*.

3) Eyes, ears and shout are large.

4) Tail length is shorter than the total length of body and head together. Tail black and uniform in colour.

5) Fur is well developed, spinous and coarse, it is black in colour.

6) Females have 6 pairs of mammary glands.

7)Body weight ranges between 1000 to 1200 gms.

V. *Mus musculus* :-

1) The genus Mus is widely distributed through out the world like *Rattus rattus*. The first molar teeth of these species are larger than the other.

2) It stays mostly in the arboreal rood burrows.

3) Elongated thin body, weighing about 15 to 35 gms. Eyes and Ears are very small.

4) Tail is very thin, sometimes equal to the total length of the head and body taken together. Rings are clearer and range from 180 to 200 in number.

5) Fur is silky brown, thin and short as compared to that of the other rats.

6) Females have 5 pairs of mammary glands.

VI. *Suncus cereleus* :-

These rodents come under Insectivora.

1) These are worldwide in distribution, found in very dirty places like streams, sewers, toilets, etc. These are nocturnal in habit.

2) Boat shaped body with triangular head and backward portion of the body.

3) Pointed snout.

4) Eyes and Ears are very small.

5) Tail is short and fleshy and delicate with long and sparsely scattered hair.

6) Fur is short, brushy and gray in colour.

7) Body weight ranges between 150 to 200 gms.

The above mentioned species of rodents were most prevalent among all of species. The rodents both dead and live after collected were identified with respect to their characteristic features and the number of each species was noted and finally a percentage of their prevalence was decided. This data was of much use since certain species are known to act as carriers for certain infections. So this data can be of a great predictive value for certain zoonotic diseases.

3. Processing of samples of dead rats for isolation of leptospires :

The freshly dead rats from those which came routinely to MRDE for plague surveillance, from different wards of the study area, were selected randomly on the basis of the post mortem changes. The rats which had post mortem changes in more extent were excluded as the body environment becomes acidic in few hours after death and the leptospires cannot survive in such environment. It was tried to collect equal number of rats from various wards, to get a even data.

The selected rats were identified on the basis of the morphology and were noted then dissected with all the possible care to avoid external contamination. Samples of tissues of kidney and liver were taken with help of sterile scissors and placed in vials containing sterile Phosphate Buffered Saline (PBS) at p^H 8. The rats were further dissected to check any visual abnormalities in the morphology of various organs.

The rats were further dissected to expose the urinary bladder's, which were checked and the residual urine if any present was collected in cryo vials with the help of sterile Pasteur pipette.

The so obtained tissue and urine samples were then transported to the laboratory for further processing.

The tissue samples were prepared by analysis by mincing them into small pieces with sterile scalpel and then homogenizing them with a mortar and pestle and the suspension was prepared by adding 9 parts of sterile PBS to it. This suspension was then centrifuged at 2600 rpm for 15 minutes. After centrifugation the supernatant was

collected in another sterile tube and the sediment which was composed of bigger tissue pieces was discarded. The supernatant was then recentrifuged at 4500 rpm for 30 minutes. This time the supernatant was discarded and the sediment was separated. A suspension was prepared from the sediment by adding sterile PBS. A wet mount of this suspension was prepared on a clean grease-free slide and Dark Field Microscopy (DFM) was performed 40 x objectives.⁽⁶⁾

The urine samples which were collected were in very small quantities so equal amount of sterile PBS was added to it, after which it was centrifuged at 1500 rpm for 20 minutes, this allowed the larger debris to settle down. The supernatant was collected in another sterile test tube and the sediment discarded. The supernatant was then recentrifuged at 5000 rpm for 20 minutes. The resulting supernatant was discarded and the sediment was screened for presence of leptospires by preparing a wet mount and observing them under 40 x objective in DFM.⁽³²⁾

After the above tests the samples which showed presence of leptospires were considered as suspected positive and were cultured in selective medium for confirmation.

4. Collection of Blood and Urine Samples from the rodents :

After finishing off the sample collection and processing of sufficient number of dead rodents. The focus of the work was shifted to the live rodents. For this the rodents were trapped from different wards of Mumbai by the MMC personnel's using Wonder traps. They were then transported to MRDE or to the rat room through the respective words.

The rodents were then taken out of the traps identified according to their morphological characteristics and noted. The rodents were then anaesthetized for this anesthetic ether was used, the rodents were placed in desiccators in which cotton soaked in anesthetic ether was added and the lid of the desiccators was placed.

Urine Collection :

The anaesthetized rodents were then hold in a pair of tongs and their abdomen was pressed, on doing so if there was any urine in bladder it came out, it was collected in sterile tubes. The pH was checked and if it was acidic it was adjusted to 8. The urine was then diluted by sterile PBS of 8 pH and the tube was then brought to the lab for further testing.

In the lab the urine diluted with sterile PBS, was centrifuged at a speed of 500 rpm for 5 minutes, and the supernatant was collected in another tube and the sediment was discarded. Due to this all the large debris and other unwanted materials like casts, etc. settled and were discarded. The supernatant was then again centrifuged at 1000 rpm for 10 minutes. After which the supernatant was discarded and the obtain

sediment was dissolved in small amount of PBS and wet mount was prepared for DGM.

Blood Collection :

After collection of urine, the anesthetized rodents were secured on a rat board to avoid their movement during the collection of blood. Once secured on rat board, cardiac puncture was performed using a 21-gauge needle of 1-inch length. For this the needle was inserted from the base of the sternum and after penetrating around 1.0 to 1.5 cms straight inside it was diverted to left towards the heart at 45° angle. At a point the heartbeat can be felt on the tip of the needle, after this needle was inserted slightly more and aspirate the piston slightly. When the blood comes in aspirate the blood as much it comes. After this the rodents were taken off from the rat board and placed back in the cage. The blood so obtained was poured in sterile tubes, which were then placed in slanting positions to let the blood clot as soon as possible. After clotting the tubes were kept at room temperature for one hour and in refrigerator at 4°C for one hour. Due to this the serum got separated, which was then stored in cryovials at 4°C until the further investigations. With 10% of sodium oxalate solution which was prepared by adding 1% sodium oxalate in phosphate buffer of pH 7.5, to avoid the blood from clotting. Then the samples were stored at 4°C until the further microscopic and serological investigations.(8) `

5. Study the Blood and Urine samples of the rodents using the following methods :

A. Microscopy :-

Leptospire are not visible through the optical bright field microscope. But when dark field is applied they show up brilliantly, presenting a appearance like 'a string of fine beads'. They demonstrate characteristic 'cork screw' motility, with their middle part held rigid and the ends bending like coat hangers. They make quick darts and rotate along the longitudinal axis.

Staining methods have been applied to increase the sensitivity of direct microscopic examinations. It is not easy to stain leptospire satisfactorily with the usual bacterial stains, but they may be demonstrated by various staining methods like silver impregnation methods of Fontana, Negative staining by using Congo red, and they can even be stained using Giemsa Stain.

I) Dark field Microscopy (DFM) :-

Dark Field Microscopy (DFM) is an indispensable aid in all leptospiral investigations; no other method of observation reveals the microorganisms so clearly in fluid medium. Due to their extreme thinness, they are practically invisible in bright-light microscopy and only slightly better results can be obtained on applying phase contrast microscopy. Any dark-field condenser of good quality may be used with a well focused source of light. The best results can be obtained if thin slides are used in combination of good and export quality glass cover slips.

For routine examinations of culture fluids, blood and agglutination tests, dry power objectives gave sufficient enlargement.

Leptospire can be easily distinguished with magnifications of approximately 200 to 300 times. But when the work demands searching for microorganisms in blood, peritoneal fluids, urine or any medium in which many other cell elements are present, then the wet mount should be observed at a higher magnification of about 400x.(33)

a. DFM of Blood Samples :-

- i) A anticoagulant solution was prepared by adding 1% sodium oxalate in phosphate buffer (pH 7.5).
- ii) 0.5 ml of this anticoagulant solution was added to 5 ml of blood sample and it was centrifuged at 3000 to 3500 r.p.m. for 15 minutes
- iii) The plasma so obtained was separated.
- iv) A wet mount of this plasma prepared and was observed using DFM.
- v) If no leptospire were seen, then the plasma sample was recentrifuged at 3000 to 4000 g for 20 minutes. The supernatant was carefully removed. And wet mount of the sediment was prepared and observed under DF.(8)

In some cases when the amount of blood sample from the rodent was very less than the serum was separated by allowing it to clot and the wet mount of this serum was prepared and the care was taken while performing DFM of blood samples, so as not to get confused with the artifacts, as when a drop of blood is observed under a cover slip under dark field illumination, thread like filaments are encountered, so it is necessary to differentiate between leptospire and the artifacts, which can be done by observing mode of motility and the ends which are hooked in leptospire.

b. DFM of Urine Samples :-

Leptospire appear in the urine in the leptospiruric phase of the infection i.e. during the second stage, that occurs after 7-10 days of the onset of infection. Leptospire get killed rapidly in the acidic urine, so immediately collection of urine the pH has to be checked and if found acidic a drop of sterile sodium bicarbonate is added to make it alkaline.

The chances of demonstrating the leptospire in urine samples increase by double centrifugation. The urine samples were processed for DFM as follows :-

i) The urine samples after adjusting the pH to alkaline were taken in sterile test tubes and centrifuged at 1800 r.p.m. for 20 minutes. This allowed the larger debris like casts to settle down.

ii) The supernatant was then transferred to another sterile test tube and recentrifuged at 5000 r.p.m. for 20 minutes.

iii) The supernatant so obtained was discarded carefully and the sediment was screened by using a DF under 40 x objective.(32)

The blood and urine samples found positive by DFM were checked further by performing staining and by using the serological techniques.

II) Staining Techniques :-

Staining of leptospire is usually not preferred for microscopy of leptospire because it is a tedious procedure. But it can be used to increase the sensitivity of direct microscopy and to make observations of its morphology. To view leptospire by bright field microscopy it is necessary to enhance its width, as they are very thin. There are

various methods of staining of leptospires like Immunofluorescence Staining, Immunoperoxidase Staining, Silver Impregnation method, Staining with Giemsa stain, negative staining by using Congo red, recently immunohistochemical methods have been applied.

Out of these all method we performed Silver Impregnation method devised by Fontana and Staining of leptospires by using Giemsa stain.

The samples which were suspected to be positive by DFM were stained only to confirm the DFM results.

a. *Giemsa staining* :-

To perform this staining, the stain mixture was prepared by diluting 0.5 ml of Giemsa stain in 10 ml of neutral distilled water.

The staining procedure is as follows -

i) A clean grease free slide was taken and smear of the sample was prepared in the center of slide by using sterile nichrome wire loop, and the smear was air dried.

ii) Then the smear was fixed by giving treatment with methanol for three minutes.

iii) The methanol was drained off and the stain mixture was flooded on the smear and allowed to act for 25 minutes.

iv) The stain was drained and the slide was gently washed in Distilled water, air dried and observed under 40 x objective.

After staining the leptospires appeared violet in color.

b. Silver Impregnation method (Fontana's method) :-

The principle of this staining method is to enhance the width of the bacterial body by deposition of silver molecules to make it visible. For this staining procedure some solutions were prepared before the staining. These solutions were -

Fixative

Acetic acid	1 ml.
Formalin	2 ml
D/W	100 ml

Mordant

Phenol	1 gm
Tannic acid	5 gm
D/W	100 ml

Ammoniated Silver Nitrate Solution

100% NH_4 solution was added to 0.5% solution of AgNO_3 in Distilled Water till the formed precipitate just dissolved. Then more AgNO_3 solution was added drop by drop till the precipitate returns and did not redissolved.

Staining :-

i) A loopful of centrifuged deposit of the material to be stained was taken, a smear was made in the center of a clean grease free slide, which was then air dried.

ii) The smear was treated with fixative for 30 seconds, this procedure was repeated for 2-3 times.

iii) The fixative was then washed off with absolute alcohol which was allowed to act on the smear for 3 minutes.

iv) The excess alcohol is drained off and the remainder is burnt of carefully until the film becomes dry.

v) The mordant was poured on the slide, and it was heated until the steam raised, it was then allowed to react for 30 seconds.

vi) The slide was then washed well in distilled water for 2-3 times.

vii) The smear was then treated with ammoniated silver nitrate, with heating till the steam raised for 30 seconds, and then the film became brown in color.

viii) The slide was then washed well in distilled water dried and checked by microscopy.

The spirochetes appeared brownish-black against brownish yellow background.

The blood samples which were treated as suspected positive samples on the basis of the results of DFM and the staining were then proceeded for confirmation of the leptospiral presence by using the serological techniques.

B. Serological Tests :-

There are various serological tests which can be performed for the diagnosis of leptospiral antibodies in the provided blood samples, few of them are, slide Agglutination Test (SAT), Microscopic Agglutination Test (MAT), Micro-capsule Agglutination Test (MCAT), Enzyme Linked Immuno Sorbant Assay (ELISA). Passive Hemagglutination Assay (PHA), Flourescence Antibody Test (FAT), Dot Elisa i.e. Dipstick Assay, Latex Agglutination Test (LAT), Polymerase Chain Reaction (PCR), etc. Few of the latest devised diagnostic tests are Dri-Dot Assay and Lateral Flow Test.

Out of all these above mentioned diagnostic tests we carried out two serological tests for confirmation of the leptospiral infection in the Rodent samples. These two tests were as follows -

I. Slide Agglutination Test (SAT)⁽¹⁰⁾ :-

Also known as Patoc Slide Test.

This is one of simplest test for diagnosis of leptospirosis still in use. It was last modified by Coghlan in 1982. Slide Agglutination Test (SAT) provides a rapid and reliable means of screening serum samples for the presence of Leptospiral antibodies. It allows a diagnosis of acute leptospirosis to be made within a few minutes of receipt of the serum sample in the laboratory.

The test was carried out on a slide which was divided into three parts with a glass marker.

Preparation of Antigen :-

a) 100 ml of liquid medium was inoculated with 1 ml of an actively growing cultures of the saprophytic strain Patoc 1. It was allowed to stand at room temperature for 7-10 days. The medium was aerated with help of a magnetic rotator so as to produce a dense culture.

b) Then neutralized formalin was added to it so as to give a final dilution of 0.5% and the culture was allowed to stand for 2 hours at room temperature so as to kill the leptospires.

c) The above mixture was then centrifuged at 11000 r.p.m. at 30 minutes. The deposited cells were then washed by resuspending them in PBS of pH 7.2, containing 1% sodium azide. This was then recentrifuged at 11000 r.p.m. The supernatant was discarded.

d) Little PBS was added to the deposit obtained, and was transferred to a screw capped bottles of 50 ml capacity, some sterile glass beads were added in these bottles. These bottles were shaken periodically for a period of 24 hrs until the suspension gave a creamy consistency.

e) These bottles were placed in large beaker containing cold water which heated upto boiling, the bottles were let in boiling water for 30 minutes.

f) After cooling the density of the suspension was adjusted to twice that of No10 on the Mc Farland scale.

g) After this the final solution was slightly centrifuged to deposit any gross particles or agglutinated leptospire if present. The supernatant was carefully separated and the pH was adjusted to 7.2.

The ready antigen was stored at 4°C until used.

Procedure of SAT :-

a) The serum sample was diluted by adding 1 drop in 9 drops of PBS in a small test tube.

b) 1 drop of the diluted serum and 1 drop of the undiluted serum samples was placed side by side in two parts of the divided slide. To each of these drops 1 drop of antigen was added and mixed carefully with help of a applicator stick.

c) The slide was then rotated on a mechanized rotator at 120 r.p.m. for 4 minutes.

d) The agglutination was read by holding the slide against dark background.

The results or the strength of agglutination was recorded as follows -

++++ = clearly defined clumps against a cleared background,

+++ = Obvious agglutination and suspension not completely clear.

++ & + = progressively weaker reactions with small clumps, sometimes formed at the periphery of the drop, and with a little clearing.

- = a negative result, when there was no change in the antigen antibody mixture.

II. Microscopic Agglutination Test (MAT)⁽⁸⁾⁽³⁵⁾⁽³⁶⁾ :-

The microscopic agglutination test (MAT) is the basis of serological diagnosis and classification. In 1926 Schuffner and Mochtar described the phenomenon of agglutination and lysis with human and animal sera and made their dilution series with drops from one and the same pipette. The reaction was read after 16-20 hours. Since then the method was modified by others like Borg-Petersen and Fagroeus in 1949, Wolf in 1954, Carbnay in 1960 and Cole *et al.* in 1973. They tried to standardize factors like incubation time, incubation temperature, reading of the end point titer and density and age of the culture.

MAT can be defined as follows -

Serial dilution of serum kept in contact with an equal volume of a well grown suspension of leptospires at a certain temperature for a certain period of time and read microscopically by estimating 50% agglutination as the end point titer of the reaction mixture.

The MAT, using live antigens, is the most widely used serological test. It is the reference test against which all other serological tests are evaluated and is the test used for import/export testing. The specificity of the MAT is good; antibodies against other bacteria usually do not cross-react with *Leptospira* to a significant extent. However, there is significant serological cross-reactivity between serovars of *Leptospira* and an animal infected with one serovar is likely to have antibodies against the infecting serovar that cross-react with the other serovars.

Antigen for MAT :-

The antigen for MAT, are the live leptospires. Here the saprophytic strain of leptospira i.e. Patoc 1, was employed, the titers of agglutination tests may be effected by the quality of the antigen and the strain used. The important criteria of a good antigen is its density, which can be estimated by spectrophotometry, darkfield microscopy and counting of leptospires.

The antigen was prepared by inoculating 10ml of liquid medium with 0.2 ml of actively growing culture of *Leptospira* saprophytic strain Patoc 1. It was incubated at room temperature for 7-10 days with aeration with the help of a mechanical stirrer or a magnetic stirrer to help to give a dense culture after the incubation period. The density of the culture was adjusted in such a way that the suspension matched the McFarland No.2 tube. On having such a density the antigen was

ready for use. In some cases even formations killed antigens have been used but live antigen gave better results. The antigen was stored at 4°C until being used. Before using it was checked for the presence of clumps of the organism if any, as it could result in the misinterpretation of the reaction and may result into a false positive result.

Test Procedure :-

a) The serum sample and the PBS were brought to room temperature before beginning the test.

b) Doubling dilutions of the serum samples was made in the wells of the microtitre plate with the help of a micropipette. 50 µl of the 1:50 diluted serum was taken in the first well of each row. 25 µl of PBS was added to all other wells. 25 µl from the first row was transferred to the second well and so on to prepare doubling dilutions ranging from 1:50 to 1:1600. One well with 25 µl of known negative and positive control was included.

c) 25 µl of the antigen was added to each well including the wells, which had positive and negative controls. One of the wells contained only the antigen to serve as a antigen control. The final dilution after the addition of the antigen ranged from 1:100 to 1:3200.

d) The antigen and the serum samples were allowed to mix by gently rotating the plate on a table.

e) After this the microtitre plate was incubated at 37°C for 2 hours.

f) After incubation, 10 µl of the mixture from the antigen control, positive control and the negative control was taken on a glass slide

and were observed with 40x objective under the darkground microscope.

g) After observation with the positive control showed clumping whereas there was no clumping in the antigen control and the negative control then the test was proceeded and the test samples were observed by 40x objective under darkground microscope.

On observation the highest serum dilution that showed approximately 50% agglutinated and 50% free leptospire was taken as the positive titer.

When the positive titer was 1:100 and below 1:150 then the process was repeated to know the exact titer as follows -

a) All the 96 wells of the microtitre plate were filled with 50 μ l PBS.

b) 40 μ l more PBS was added to the wells of the column 2.

c) 10 μ l of the serum sample was added to the wells of the column 2, by doing so the dilution of the serum was 1:10.

d) A serial dilution was performed by transferring 50 μ l from the first column to the second and so on. The final 50 μ l was discarded.

e) After this 50 μ l of the antigen was added to all the wells. Due to this the dilution in the wells of the second column was 1:20 and 1:40 in the third and so on.

f) The plate was then isolated gently on the table so as to allow the serum and the antigen to mix.

g) The plate was then incubated for 2 hours at 37°C.

After incubation the process of observation of the agglutination was performed as done earlier, and the highest dilution giving 50% agglutination was considered as the antibody titer of the serum sample.

The serum and the urine samples which were positive by the Microscopy and the serological tests were inoculated in the selective media for the isolation of the causative organism. The media used is available in commercial dehydrated form. The nutritional requirements of leptospire and the composition and type of the selective media used for the culturing process are as follows -

C. Blood and Urine Culture in selective media for Leptospira :-

The nutritional requirement of leptospires are unique although simple. Vit B₁ and B₁₂ and long chain fatty acids are the only organic compounds that are known to be required. Fatty acids are there source of energy and carbon, and are required as a source of cellular lipids since leptospira cannot synthesize fatty acids denovo. Due to the inherent toxicity of free fatty acids, these must be supplied to the leptospires either bound to albumin. Carbohydrates are not a suitable source of energy or carbon. Even though amine acids are utilized to a limited extents they cannot satisfy the nitrogen requirements of these organisms.⁽³⁷⁾

The non essential nutrient, pyruvate, enhances the initiation of growth of the fastidious leptospires.

Leptospires incorporate purine bases but not pyrimidine bases into their nucleic acids. Because of this they are resistant to the antibacterial activity of the pyrimidine analogue, 5-fluoro-uracil. This compound is used in selective media for the isolation of leptospires from contaminated sources.⁽³⁷⁾

The media used for the cultivation and isolation of leptospires can be categorized in three types –

I. Liquid Media :-

Essential for isolation of leptospire and growing cultures to be used as antigen in agglutination tests as the pieces of agar which are present in the semi-solid media interfere with the interpretation of the results. Growth of leptospire in liquid media is indicated by the increase in turbidity, granular appearance or deposit formation in the media.⁽³⁷⁾

II. Semi-solid Media :-

This type of media contains 0.1-0.5% Agar (w/v). Semi-solid media is preferred for isolation of strains and for maintenance of strains. Growth in these media is easily visualized in form of rings of dense growth, which is several mm to cm below the surface of media. Semi-solid media in screw capped tubes are generally used for maintenance of stock cultures.⁽³⁷⁾

III. Solid Media :-

These media contains 0.8 to 1.3% of agar (w/v). The lower the concentration of agar, the more likely is the growth tendency of leptospire to swarm across the plate and through the medium; the higher the agar concentration, smaller are the colonies. The growth occurs below the surface, plates have to be sealed to prevent dehydration. This method is used for isolating strains from contaminated natural materials, contaminated cultures or cloning of mixed leptospiral infections. The colonies in 1% agar are subsurface and become visible within 7-14 days in most serovars.⁽³⁷⁾

Growth Requirements of Leptospires⁽³⁷⁾ :-

There are various compounds which are required for the growth of leptospires in the media. The compounds and the requirements which they fulfill are as follows -

Peptone - Protein source.

Asparagine - Can replace peptone.

Beef Extract - Protein source.

Fatty acids - *Leptospira* growth depends on long chain fatty acids for energy and carbon source.

Sodium pyruvate - Addition of 50-400 µg/ml of sodium pyruvate is optimal for reducing the lag periods seen with lower inoculum.

Glycerin - Addition of 50-400 µg/ml of glycerol decreases the generation times and increases the growth rates.

Salts - Higher salt concentrations might be essential for membrane stabilization and growth of cells.

Na/K - Stimulatory effects of higher levels of Na and K are seen on the growth. The requirements for higher K could be replaced by equimolar amounts of Na although the use of K permitted shorter generation times.

Ca/Mg - Virulent cells respond quickly to the higher levels of Mg. Increased levels of Ca did not replace the requirements for higher levels of Mg.

Fe 2+ - Iron is known to be active in oxidation-reduction functions and may contribute to the reduction of peroxides which results from the accumulation of organic proxides resulting from auto-oxidation of long chain, unsaturated fatty acids. Fe sulfate solution should be freshly prepared. Iron is not only an adequate substitute for hemoglobin but also retains its effectiveness longer.

Ammonium- Ammonium salts are an effective source of cellular nitrogen.

Rabbit Serum - The growth of leptospires was often improved when rabbit serum was slightly hemolysed. Increasing percentage of serum upto and including 12% results in a progressively steeper growth curve. This stimulation is seen in the first four days and is significant in attainment of high early growth levels. 6-8% of rabbit serum enrichment results in total growth response is linear upto 6%.

Fluoro-Uracil - Uracil is a pyrimidine, and pyrimidine when combined with fluorine, it becomes lethal to various micro-organisms, but not to leptospires as it does not requires pyrimidine from external source. 5 FU is added in small amounts like 200-500 $\mu\text{g/ml}$ this make it selective media.

It is generally assumed that animal serum is one of the most important growth factors for culture of leptospires. Most of the media described require rabbit serum as the protein source. In cultivating leptospires one of the most striking features is the irregularity in growth pattern according to the kinds of media used.

The media used for the cultivation and isolation of leptospires in the laboratory for this work were as follows -

I. LIQUID MEDIA :-

a) Ellinghausen and McCullough (EM) medium for Leptospire⁽⁸⁾⁽³⁸⁾

This media was modified by Johnson and Harris in 1967, so it is known as EMJH media. The reagents that are required for the preparation of EMJH are as follows :-

Phosphate buffer solution.

Na_2HPO_4	16.6g
H_2PO_4	2.172g
Distilled water	1 litre

Salts Solution.

NaCl	38.5g
NH_4Cl	5.35g
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	3.81g
Distilled water	1 litre

Copper Solution.

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	30 mg
Distilled water	100ml

Zinc Solution.

$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	80mg
Distilled water	200ml

Iron Solution.

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	500mg
Distilled water	200ml

Vitamin B₁₂

Concentrated stock : 10 mg in 100 ml of Distilled water

working stock : 10 ml concentrated stock in 90 ml of D/W.

Thamine hydrochlorite (stock solution).

200 mg in 100 ml distilled water.

Tween 80.

10 ml of Tween 80 was dissolved in 70 ml distilled water at 60°C, adding drop by drop, the volume was adjusted to 100 ml and then further diluted to 1000 ml. This 1% solution was stored at -60 until used.

Bovine albumin.

5% albumin solution was prepared by dissolving 5 g bovine albumin in 100 ml of phosphate buffer solution. The pH was adjusted to 7.4 by adding NaOH, and then sterilized by membrane filtration through membrane of 0.22 µm pore size.

Preparation of Medium :-

1. 700 ml of distilled water was taken to which following things were added-

Phosphate buffer solution	40 ml
Salts solution	50 ml
Copper Solution	1 ml
Zinc solution	10 ml
Iron solution	20 ml

The mixture was shaken for 5 minutes.

2. 200 mg L-cystine was added, the mixture was shaken for 3 minutes. This was filtered through Whatman No.1 paper.

3. After this following things were added.

Vitamin B ₁₂ (working stock)	20 ml
Thiamine stock solution	0.1 ml
Tween 80 (1% solution)	120 ml

The total volume was adjusted to 1 liter. This basal mixture was dispensed about 4-8 ml in tubes and these tubes were autoclaved at 121°C for 15 minutes.

4. The above media was then cooled and sterile bovine albumin solution was added in proportion of 1 volume to 4 volumes of basal medium. The resulting media was sterilized by filtration through a sandwich of membrane filters of pore sizes 1.4 µm, 0.45 µm and 0.22 µm.

The above media was then checked for sterility by placing few randomly selected tubes at room temperature for 48 hours to check any bacterial growth if present. The remaining media was stored at 4°C until used.

b. Leptospira Medium, Base :-

This is one of the liquid media which is available in dehydrated form and is provided by Himedia.

To prepare this media 0.256 gms. of the dehydrated media powder was added in 100 ml of sterile distilled water and then it was sterilized by autoclaving at 121 lbs pressure for 20 minutes. After which it was allowed to cool.

10 ml Rabbit serum was filter sterilized by passing it through membrane filter of pore size 0.22 μm . This serum was then added to the medium base. And the media was dispensed in tubes with screw caps in 6-8 ml volumes. Tube media was checked for sterility by placing few randomly selected tubes at room temperature for 48 hours.

After checking the sterility of the medium, 25 μg of 5-flurouracil per 100ml of medium and 0.2 μg of Neomycin sulfate per 100 ml of the media to avoid any unwanted bacterial and fungal contamination.

On inoculation, the growth of leptospires in the media was detected on the basis of to increase in turbidity.(8)(32)(37)(38)

II. SEMI-SOLID MEDIA :-

The semi-solid media that can be used for the cultivation of leptospires are Fletcher's Medium, Bovine Albumin Tween 80 semisolid Medium, Bovine Serum Albumin Tween 80 Soft Agar etc. Out of all these media we used the Fletcher's medium which was available in dehydrated form, from the Himedia.

Fletcher's media, a semisolid agar is suitable for isolating strains of leptospires and for maintaining them for many months. They multiply within the upper part of the tube forming zones of turbidity, which are known as Dinger's rings.

The chemical composition Fletchers Agar -

Peptone	-	0.3 g
Beef extract	-	0.2 g
NaCl	-	0.5 g
Agar	-	1.5 g
Distilled water	-	920 ml

All the above ingredients were mixed and dissolved with help of boiling. It was then sterilized by autoclaving at 121°C for 20 minutes. Cooled to 50°C and then filter sterilized rabbit serum was added in 10% volume of that of the media. This media was then dispensed in 8-9 ml volumes in screw capped tubes. Sterility was checked by placing few tubes at 37°C for 48 hrs.

25 µg of 5-flourouracil and 0.2 µg of Neomycin sulfate was added per 100 ml of media prior to dispensing it into the tubes, to avoid any unwanted bacterial and fungal growth.(8)(32)(37)(38)

III. SOLID MEDIA :-

The solid media used to check the growth of leptospire was the Tryptose-Phosphate agar which was first used by Cox and Larson in 1957.

Composition of media

Tryptose-phosphate broth	-	0.2 g
Agar	-	1 g
Distilled water	-	90 ml

The pH of the above mixture was adjusted to 7.5 and then is was sterilized by autoclaving at 121°C for 15 minutes. After cooling 10 ml of filter sterilized rabbit serum was added. To this 1 ml of hemoglobin solution was added. The whole mixture was then heated in water bath at 56°C for 30 minutes. After this the media was poured in Petri dishes to give a depto of 6-8 mm.

After inoculation the plates were sealed with help of parafilm to avoid drying of the media.⁽⁸⁾⁽³⁹⁾

IV. PREPARATION OF SAMPLES FOR CULTURING⁽⁸⁾:-

a. Blood Samples :-

(1) The collected blood samples which were suspected positive was inoculated in tubes containing liquid media in varying amounts ranging from 1 to 4 drops of blood and centrifuged.

(2) After centrifugation the deposit was resuspended in 2 ml PBS.

(3) The resulting suspension was then inoculated in liquid, semisolid and solid media.

(4) All these were then incubated at room temperature, and was examined periodically for leptospiral growth evidence with help of microscopy under DF.

(5) This was carried out for atleast 6 weeks before discarding the samples as negative.

b. Urine sample :-

The urine samples that were suspected as positive on the basis of DFM were processed same manner as that with blood samples and were inoculated in media of all three types and incubated at room temperature. These were examined periodically for 5-6 weeks by using DFM for any leptospiral growth before being discarded as negative.

(6) Culture isolation and confirmation of the results with respect to the standards.

After performing all the basic and the serological tests the samples which gave the positive results were cultured in selective leptospira media. The growth pattern, morphology etc. of these isolated leptospire was then compared to that of the standard strains, which were also maintained in the same selective media. The comparison was done by observing the growth pattern in semisolid media and by comparing the morphology with help of DFM. The leptospire isolated from the samples were maintained in the selective media with regular subculturing.

RESULTS

RESULTS :

(1) Collection of Rodents from the Mumbai City and its suburbs :-

A. Dead Rodents -

The collection of dead rodents was done, by keep in mind to avoid those rodents which had undergone severe post mortem changes. And they collected randomly, trying to include the samples from every ward.

In the above manner a total of one hundred twenty (120) dead rodents were screened for the presence of leptospires.

B. Live Rodents -

As already mentioned, the live rodents were trapped and transported to us by the MMC staff. The rodents which had died during the transportation were excluded from the study.

In this way a total number of two hundred forty (240) live rodents were used for the detection of leptospires.

(2) To study the prevalence of various rodents species in the study area :-

As mentioned already the rodents both dead and live were identified with respect to their morphological characters, and the number of individual species was noted and the species wise distribution data was drawn separately for the dead and live rodents, it was as follows -

A. Dead Rodents -

Out of the total of one hundred twenty (120) dead rodents collected for the screening for the presence of leptospire, the species wise distribution was as follows -

Species	Percentage	No.of Rodents
<i>Rattus rattus</i>	56.6%	68
<i>Rattus norvegicus</i>	15.0%	18
<i>Bandicota bengalensis</i>	23.4%	28
<i>Mus musculus</i>	5.0%	06

B. Live Rodents -

Out of the total of two hundred forty (240) live rodents collected from different parts of the study area for screening of presence of Leptospire, the species wise distribution was as follows :-

Species	Percentage	No.of Rodents
<i>Rattus rattus</i>	28.4%	67
<i>Rattus norvegicus</i>	19.3%	46
<i>Bandicota bengalensis</i>	47.2%	112
<i>Bandicota indica</i>	2.5 %	6
<i>Mus musculus</i>	3.2 %	8
<i>Suncus cereleus</i>	0.4 %	1

(3) Processing of samples of dead rodents for isolation of leptospires -

Out of the total of 120 dead rodents the tissues like kidney and the urine samples if found were taken and were checked by DFM for presence of leptospires. On performing DFM one tissue sample showed presence of leptospires. The discription of the sample was as follows -

Species	Area
<i>Rattus norvegicus</i>	S ward (Bhandup)

But later when the above sample was inoculated in selective media both liquid and semi-solid, it produced no detectable growth of leptospires for several weeks. So the sample was finally considered as negative.

(4) Collection of Blood and Urine samples from the live rodents –

Out of the total of 240 live rodents, only from 182 rodents the collection of urine sample was possible. Blood samples were obtained from all the 240 rodents.

(5) Study of the blood and urine samples of the live rodents -

A. Urine Samples :-

The 182 urine samples which were obtained from the 240 live rodents, were first screened for the presence of leptospire by using the DFM, the results of which are as follows -

Two urine samples out of 182 samples were found to be positive for leptospiral presence by DFM, these were -

Species	Area
<i>Bandicota bengalensis</i>	D ward (Nana Chowk)
<i>Bandicota bengalensis</i>	E ward (Byculla)

The above positive samples were then cultured in the liquid medium and after the incubation of 8 days the growth was confirmed to be of leptospira by using DFM.

B. Blood Samples -

The 240 blood samples which were obtained from the 240 live rodents were processed for separation of serum, which was first screened for the presence of leptospire by performing DFM using a 40 x objective. The results of DFM were as follows -

DFM - Out of the total of 240 samples 6 were positive for the presence of leptospire by DFM these were as follows.

Species	Area
<i>Rattus rattus</i>	G/n (Dadar Plaza)
<i>Rattus norvegicus</i>	G/n (Dadar Plaza)
<i>Bandicota bengalensis</i>	L (Kurla)
<i>Rattus norvegicus</i>	D (Nana Chowk)
<i>Rattus norvegicus</i>	E (Byculla)
<i>Rattus rattus</i>	E (Byculla)

The above 6 samples were considered as suspected positive and were then proceeded for the confirmation by using the serological tests which were Slide Agglutination Test (SAT) and Microscopic Agglutination Test (MAT).

SAT - On performing SAT for the 6 samples which suspected to be positive with the help of DFM, the results were as follows -

Species	Area	Agglutination
<i>Rattus rattus</i>	G/n	-
<i>Rattus norvegicus</i>	G/n	+
<i>Bandicota bengalensis</i>	L	-
<i>Rattus norvegicus</i>	D	+++
<i>Rattus norvegicus</i>	E	++
<i>Rattus rattus</i>	E	+++

As indicated, 2 of the 6 suspected positive samples by DFM, gave negative results for SAT. The remaining 4 samples were then proceeded for MAT, to estimate the antibody titre in the infected rodents.

MAT - MAT was performed on the 4 samples which were positive for SAT. The antibody titre of these samples by MAT was as follows -

Species	Area	Agglutinating Titre
<i>Rattus norvigicus</i>	G/n	1:80
<i>Rattus norvegicus</i>	E	1:100
<i>Rattus rattus</i>	F	1:120
<i>Rattus norvegicus</i>	D	1:120

Hence by performing MAT, which is considered to be the gold standard method by diagnosis of Leptospirosis, 4 rodents were found to be present in acute stage of infection.

**DISCUSSION
AND
CONCLUSION**

DISCUSSION AND CONCLUSION

Leptospirosis, being one of the most common zoonotic infection with a worldwide presence, has always been a great danger and of a great economic importance to both the mankind and the wildlife. In developed countries, leptospirosis continues to be a disease of considerable economic significance in animal husbandry, whereas in the tropical and subtropical countries it has caused the major burden of human disease. Several recent outbreaks of leptospirosis from almost every part of the world have drawn attention to the potential effects of climate change and human activity on the incidence of the disease and broad spectrum of clinical manifestations.

Keeping in mind the recent outbreaks of leptospirosis in the study area and the loss which both the mankind and the animals had to face during the recent past, and the role of rodents in transmitting the infection to most of the animals and humans, which has given it the status of a root source of infection. We decided to undertake this study. Our principal objective of this study was to investigate the status of leptospiral infections in rodents and to help us to predict the future possible outbreaks by knowing the places where the rodents were having the infection either in the acute stage or were in carrier stage. This information would be of great advantage so as to reduce the threat which the disease poses for the mankind and the animal life.

The first factor which we surveyed were the past outbreaks and their locations. The areas which were considered to be endemic and had few successive outbreaks were surveyed. The environmental conditions like drainage facilities, the environmental pH and the animal density and variety was observed. Then efforts were made to obtain as much possible sample from such locations. Even the environmental samples like soil, water were screened for the presence of leptospire if any.

After checking the environmental conditions the rodent population and species wise distribution was checked for the localities. The areas which had high rodent density and the supportive environmental conditions for the spread of leptospira were given preference. The rodents were collected from all the wards to get the overall status of leptospiral infection.

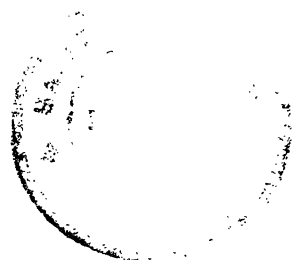
A total of 120 dead rodents were collected, which represented almost all the 24 wards of the study area. The tissue samples like kidney of these rodents were taken and checked for leptospiral presence. Out of the total 120 rodents, only one gave positive results by DFM, but it was also later considered as negative as the sample did not show any growth in the selective media.

After dead rodents, the live rodents were collected for leptospiral screening. A total of 240 live rodents were collected. Out of these 240, 182 urine samples were obtained 2 urine samples, both of *Bandicota bengalensis* belonging to D ward i.e. Nana Chowk and E ward i.e. Byculla were found to be positive for leptospiral presence and these were successfully cultured in selective media. And from 240 blood samples, 6 were positive by DFM, which on performing SAT only 4 samples were positive. MAT was performed by taking these 4 samples and their antibody titer was noted. These were 3 *Rattus norvigicus* and one *Rattus rattus* from G/n, D, E and E wards respectively. The antibody titers were 1:80, 1:100, 1:120 and 1:120 respectively.

With the above results it is confirmed that 2 of the 240 live rodents were carriers and 4 were in acute stage of infection.

In this way we can use the above results to predict the chances of any future outbreak in the areas where the rodents were found to be having the leptospiral infection or were in carrier stage.

BIBLIOGRAPHY



BIBLIOGRAPHY

1. Faine S., 1998. Leptospirosis, pg 849-869, In Topley and Wilson's Microbiology and Microbial Infections, Vol.3, 9th edition, Arnold Publication.
2. Levett P.N., 2001. Leptospirosis, pg 296-326, Clinical Microbiological Reviews, Vol.14, No.2.
3. Christie, 1969. Leptospiral Infections, pg 873-895, In Infectious Diseases, Epidemiology and Clinical Practices, Livingstone Publication.
4. Muthusethupathi M.A., 2000. Leptospirosis, It there a need for Concern ?
<http://www.caving.org.uk/wdic/madras2000>
5. Faine S., 1991. The Genus Leptospira, pg 3568-3582, In the Prokaryotes, Vol.4, 2nd edition, Springer Verlag Publication.
6. Robbin S. Weyant *et al.*, 1999. Leptospira and Leptonema, pg 739-743, In Mannual of Clinical Microbiology, 7th edition, ASM press.
7. Faine S., 1998. Leptospira, pg 1287-1302, In Topley and Wilson's Microbiology and Microbial Infections, Systematic Bacteriology, Vol.2, 9th edition, Arnold Publication.
8. Joyce D. Coghlan, 1996. Leptospira : Borrelia pg 661-674, In Mackei Mc Cartney's Practical Medical Microbiology, 14th edition churchill Livingstone Publication.
9. Simbert R.M., 1977. Leptospira, pg 216-228, In CRC Handbook of Microbiology, Vol. 1, 2nd edition, CRC press.

10. Levett P.N., 1997. Leptospirosis, pg 677-690, In Principles and Practices of Clinical Bacteriology, John Wiley and Sons.
11. Judith Green - Mc Kenzie, Leptospirosis, www.emedicine.com
12. Blood D.C. *et al*, 1979. Diseases caused by *Leptospira* spp., pg 565-574, In Veterinary Medicine, 5th edition, Tindall (ELBS) Publication.
13. Sehgal S.C., 1998. Emergence of Leptospirosis as a Public Health Problem, pg 7-16, In Leptospirosis, Proceedings of the Third Round Table Conference. Ranbaxy Science Foundation.
14. Fatal Leptospirosis, Azores Islands, 2001. pg 109-112, In Weekly Epidemiological Record (WHO), Vol. 76 No.15.
15. <http://www.ce.berkeley.edu/~nelson/ce210a/Leptospira/leptospira.htm>
16. Paul V. Effler *et al.*, 2002. Evaluation of Eight Rapid Screening Tests for Acute Leptospirosis in Hawaii, pg 1464-1469, Journal of Clinical Microbiology, Vol.40, No.4.
17. Leptospirosis, Worldwide, 1999. pg 237-242, In Weekly Epidemiological Record (WHO), Vol. 74, No.29.
18. <http://www.dailynews.lk/2003/10/15/new23.html>.
19. J. Singh and J. Sokhey, 1998. Epidemiology, Prevention and Control of Leptospirosis, pg 17-32, In Leptospirosis, Proceedings of the Third Round Table Conference, Ranbaxy Science Foundation.
20. Leptospirosis, India, 2000. pg 217-223, In Weekly Epidemiological Record (WHO), Vol. 75, No.27.

21. B. Srinivas Kakkilaya, *et al.*, 2000. Investigation of Leptospirosis Outbreak in Surathkal, A Poster presented at Oxford 2000 - New Challenges in Tropical Medicine and Parasitology Conference.
22. Rama Chaudhary *et al.*, 2002. Emerging Leptospirosis, North India,
<http://www.cdc.gov/ncidod/EID/Vol.8No.12/02-0052.html>.
23. <http://www.mumbai-central.com/grapevine/msg00575.html#3>.
24. <http://newsarchives.indianfo.com/2000/08/17/17/disease.html>.
25. <http://www.trustmed.com.tus/news/2002/08/04/20000803059.e.html>.
26. Sushil Shah and Nilesh Shah, 2003. Cross Reactivity Observed in Patients whose Blood samples tested positive for *Leptospira* IgM and Dengue IgM antibodies, pg 284-288, Bombay Hospital Journal, Vol. 45, No.2
27. L.S. Iyer, 1973. Studies in Leptospirosis, Ph.D. Thesis, Haffkine Institute.
28. R. Verma and S.K. Srivastava, 1998. Leptospirosis in Animals : prevalence and distribution, pg 48-63. In Leptospirosis, Proceedings of the Third Round Table Conference, Ranbaxy Science Foundation.
29. Alex J.C. *et al.*, 1993. *Leptospira* Isolates of Madras City, pg 269-273, Indian Journal of Medical Microbiology, Vol. 17, No.4.
30. Zamora J. and Reidemann S., 1999. Isolation and Survival of *Leptospira* in Kidney tissues of Wild rodents. pg 103-107, Archives of Medical Veterinary Vol. 31, No.1.

31. N.L. Gangadhar *et al.*, 2000. Reservoir Hosts of *Leptospira inadaï* in India, pg 793-799, Rev. Sci. Tech. Off. Int. Epig., Vol. 19, No.3
32. Leptospira Workshop, 2000. B.J. Medical College, Pune.
33. J.W. Wolf, 1954. The Laboratory Diagnosis of Leptospirosis, Charles C. Thomas Publication.
34. B. Babudieri, 1961. Laboratory Diagnosis o Leptospirosis, pg 45-58, Bulletin of World Health Organization, Vol. 54.
35. H. Korver, Microscopic Agglutination Test (MAT) for the Diagnosis of Leptospirosis and serotyping of Leptospire, <http://tesla.rcub.bg.ac.yu/~lepto/lab/mat.html>.
36. Leptospirosis, Manual of standards for Diagnostic tests and vaccines, 4th edition 2000. http://www.oie.int/eng/normes/mannual/A_00041.htm.
37. Nutritional Requirements for the growth of Leptospire. <http://tesla.rcub.bg.ac.yu/~lepto/lab/media.html>.
38. Ronald M. Atlas, 1993. Handbook of Microbiological Media, CRC Press.
39. Cox and Larson, 1957. Colonial Growth of leptospira, pg 578 – 79, Journal of Bacteriology, Vol. 73,
40. Leptospirosis and your Pet. http://www.cdc.gov/ncidod/dbmd/diseaseinfo/leptospirosis_g_pet.htm
41. A.S. Ahl, D.A. Miller, P.C. Bartlett, 1992. Leptospira serology in small ruminants on St. Croix, U.S. Virgin Islands Pg 168 – 171, Annals New York Academy Of Sciences, Vol. 16, No. 653

42. Roger Marshall, Review of Canine Leptospirosis in New Zealand.
<http://www.infobrok.co.nz/canine.htm>
43. Roger Marshall, Shedding of Leptospire by Cattle.
<http://www.infobrok.co.nz/shed.htm>
44. Recommendations to Veterinarians on Leptospirosis Eradication in Grower pigs.
<http://www.infobrok.co.nz/pglepto.htm>
45. Donald B. Hudson, Leptospirosis in Domestic Animals.
<http://www.ianr.unl.edu/pubs/animaldisease/g417.htm>.
46. Ithaca N.Y., Canine Leptospirosis.
<http://www.dnet.net/personal/cotrell/lepto.htm>.

SYNOPSIS

**A SYNOPSIS OF THE THESIS TO BE SUBMITTED TO THE
UNIVERSITY OF MUMBAI FOR THE DEGREE OF
MASTER OF SCIENCE IN APPLIED-BIOLOGY**

Title of thesis : To study the prevalence of leptospirosis in rodent population in Mumbai and nearby areas.

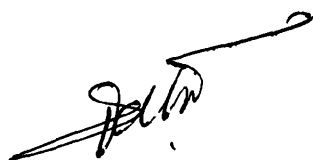
Name of the candidate : Mr. Vikrant H. Tripathi B.Sc.

Name and designation of the Guide. : Dr. B. D. Sathe Ph.D.
Ex. Assistant Director and Officer – in – Charge,
Dept. of Zoonosis, Haffkine Institute.

Place of research : Dept. of Zoonosis, Haffkine Institute,
Parel, Mumbai – 12.

Number and date of Registration. : 51 / 28 Nov. 2001.

Signature of the Guide.



Dr. B. D. Sathe,
Ex. Assistant Director,
Officer – in – Charge,
Dept. of Zoonosis,
Haffkine Institute.

Signature of the Candidate.



Vikrant H. Tripathi

INTRODUCTION:

Leptospirosis, which is considered as one of the most common zoonosis in the world, is a ubiquitous zoonosis, which affects both humans and animals. ⁽¹⁾

It is caused by the spirochete *Leptospira*, the name *Leptospira* which was proposed by Noguchi in 1918 after a detailed microscopic and cultural observations, is derived from Greek language where “Leptos” means “thin” or fine, and “Spira” means a “coil” and so on the whole it means ‘finely coiled’. ⁽¹⁾

Leptospiral infections are found in a very wide host range including the warm blood vertebrates, mammals, marsupials, certain amphibians, even birds in some cases and even found free living in soil and surface waters. There are many synonyms to leptospiral infections like Weil’s disease, Swineherd’s disease, Canicola fever, Canefield Leptospirosis, Swamp or Mud fever and Indonesian Weil’s disease. ^(1,3)

Most leptospiral serovars have their primary reservoirs in wild animals, which continually reinfect domestic populations. The causal organism affects at least 160 mammalian species and has been recovered from rats, bandicoots, swine, dogs, cats, raccoons, cattle, and other animals, out of which rodents are most common source worldwide. ^(7,8,9)

Urinary shedding of infected animals is the most important source of the bacterial pathogens. Contact with the organisms via infected urine, contaminated abortions or urine contaminated media results in human infection. The organism enters the body via abraded skin or mucus membranes. After gaining entry into the body, the organism multiplies in the blood and tissues causing leptospiremia, but main target organs are liver and kidney. After gaining access to kidney it produces interstitial nephritis and tubular necrosis finally leading to renal failure. Liver involvements results into centrilobular necrosis leading to jaundice, other organs if invaded may result into several other symptoms edema, vacuolization of myofibrils, focal necrosis, fluid leakage, and recurrent or chronic uveitis. ⁽¹⁾

Out of the total human infections around 30 to 50% result from occupational exposure. The high risk group include farm workers, veterinarians, pet shop owners, field agricultural workers, milkers, plumbers, sewer workers, meat handlers, slaughter house workers, coal miners, workers of fishing industry and military troops. ^(1,2,4)

As termed zoonosis it originates from the animals, among which the primary reservoirs are the rodents because, once infected they shed the organisms via urine for the lifetime. This in turn infects the other animals

and humans. Other animals that act as reservoir hosts are dogs, cattle, pigs, horses, etc. in short all the animals can be infected. ^(4,8)

The signs of leptospiral infection in animals may range from fever, malaise, loss of appetite, haemoglobinuria, weakness, jaundice, nephritis, etc. The abortions, failure to thrive, loss of milk yield, costs of vaccine development and vaccination, loss of commercial livestock makes this infection of a great Economic importance. ^(3,10)

Leptospirosis has been isolated from approximately 160 mammalian species in the temperate zone. It has been isolated from bandicoots and rats in Madras city in the year 1993. ^(7,9)

Leptospirosis is prevalent in India in many areas right from 1931, when Taylor and Goyal first reported it from Andaman Islands ⁽⁶⁾. Since then many outbreaks have reported till present few of which are as follows –

1. 1993	Tamil Nadu	Ratnam et. al. ⁽⁹⁾
2. 2000	Mumbai	www.trustmed.com ⁽¹¹⁾
3. 2000-01	Delhi	Chaudhary et.al. ⁽¹²⁾
4. 2003	Mumbai	Sushil Shah ⁽¹³⁾

Among these WHO have reported few, in one it has reported in 1999 Weekly Epidemiological Records (WER) July issue, they have given detailed data of the positive cases found worldwide, according to the reports the number of cases in India was 2664 out of which 1679 were males and 985 were females.

And in WER. of July 2000, they have reported the outbreak, which took place in Orissa during the post-cyclone period in November 1999. During that time around 142 samples were collected from 4 different villages and few were collected from the hospital, out of these 86 were negative and 56 were positive.

And in one of the most recent reports, by Sushil and Nilesh Shah of Bombay hospital, they have reported 481 samples positive, these samples were collected during the year 2002-2003 from Mumbai city and its suburbs. ⁽¹³⁾

Hence it was necessary to undertake the following project to find the status of the reservoir and root source of infection and its prevalence in Mumbai city and its suburbs and also to enable us to predict the location of future outbreaks.

AIMS & OBJECTIVES:

1. Collection of rodents from the Mumbai city and its suburbs.
2. To study the prevalence of various rodent species in the study area.
3. Processing of samples of dead rats for isolation of *Leptospira*.
4. Collection of Blood and Urine samples from the rodents.
5. Study the blood and urine samples of the rodents using following methods-
 - a. Microscopy.
 - i. Dark Field Microscopy (DFM).
 - ii. Staining techniques.
 - b. Serological tests-
 - i. Slide Agglutination Test (SAT)
 - ii. Microscopic Agglutination Test (MAT).
 - c. Blood and Urine culture in selective media for Leptospirosis.
6. Culture isolation and confirmation of the results with respect to the standards.

MATERIALS & METHODS:

1. Collection of rodents from the Mumbai city and its suburbs:

The collection of rodents was carried out with the help of Municipal Rat Destruction Establishment (MRDE), a unit of Mumbai Municipal Corporation (MMC) and the Pest Control Officers (PCO) of different wards of Mumbai. The rodents collected by the MMC staff at the MRDE were used.

In the first part of the work only dead rats were used for the investigations. Rats killed by the MMC staff for routine plague surveillance work; were used for leptospiral diagnosis in the lab. In the second part, live rats were also collected from MMC and MRDE using traps.

The traps and cages used for trapping and maintaining the rodents were obtained from MMC and Dept. of Zoonosis.

Traps used for the above purpose were of three types i.e.

1. Wonder traps were used for trapping all kinds of rats due to its big size.
2. Medium sized wonder traps were used for trapping *Rattus norvegicus* and *Rattus rattus*.
3. D – cages were used for maintaining live rats in the laboratory.

2. To study the prevalence of various rodents species in the study area:

Survey of the endemic areas of Mumbai was conducted with reference to various species of rodents found in that area, and the resultant data was analyzed to obtain species distribution of various rat species in the study area.

This data useful since certain species of rodents are specific to certain infection, and therefore can have predictive value in epidemiology of Zoonotic diseases.

3. Processing of samples of dead rats for the isolation of Leptospire:

The freshly dead rats received from the different wards were selected randomly and dissected to obtain tissue samples of kidney and liver. Tissues were collected in sterile Phosphate Buffered Saline (PBS) of pH. 8, while doing so care was taken to avoid the external contamination. The rats were dissected and checked for any abnormalities in the morphology of the organs.⁽¹⁴⁾

The urinary bladders were checked and any residual urine if present was collected with the help of a sterile Pasteur pipette in the cryo vials.

The samples were transported to the lab. The tissues were homogenized in PBS for examination using Dark field Microscopy. This was conducted on urine samples after noting and adjusting its pH to 7.2 and then diluting it with PBS and centrifuging.

4. Collection of blood and urine samples from the live rodents.

Rats were trapped from different locations of Mumbai city and its suburbs using the wonder traps by the MMC staff and were then transported either to the MRDE unit or the lab directly.

These rats were then anesthetized and processed for sample collection as above.

Urine Collection:

For urine collection the rats were held with a pair of tongs and urine was collected by pressing the abdomen. The urine was collected in tubes. The pH was noted and if required was adjusted to 8. Urine was centrifuged at 500 rpm for 5 minutes and the sediment was discarded. The supernatant was recentrifuged at 800 to 1000 rpm for 10 minutes. The supernatant was discarded and the sediment was redissolved in PBS of pH 8 for microscopic examination.^(5,14)

Blood Collection:

For blood collection anesthetized rats were secured on rat board and blood was collected by cardiac puncture technique using a 21 gauge x 1 " needle. The blood so obtained was stored at room temperature for one hour in tubes and then for the same period at 4 °C. The serum was separated and stored in cryo vials and at 4 °C till further investigations.⁽⁵⁾

5. Blood and urine samples obtained from rodents were studied using the following methods.

The blood and urine samples collected from the rodents were processed for isolation of the leptospires as under:–

a. Microscopy:

i. Dark Field Microscopy (DFM).

For detection of leptospires in the given samples the DFM was applied as it is the best option available with us to detect the live organisms from the sample and it was necessary to study few of the characteristic properties of *Leptospira*, like their ends which are normally hook shaped, their cork-screw type of motility and their spiral morphology.

Microscopic examination of the samples was performed as follows:

Blood: One drop of serum was mounted on a clean slide and a cover slip was placed over it and was observed under DFM at 40 x and if no organisms were seen then the sample was recentrifuged at a higher speed like 3000 to 4000 rpm for 20 minutes and DFM was performed again. ⁽¹⁵⁾

Urine: The urine samples were examined by DFM for the detection of leptospires. The urine samples were serially diluted with PBS to reduce the contaminants. ⁽¹⁵⁾

Care was taken while doing the microscopy so as not to confuse between the artifacts like lysed RBC's while observing the blood samples, whereas same was done with urine to avoid considering artifacts like casts, etc. which are found abundantly in urine.

ii. Staining Techniques.

Leptospira staining is not a commonly used method as these are best viewed by DFM and also it is the most preferred method as it does not require any staining and is very easy to perform in a very short period of time. Where as staining is a very complex and time consuming procedure and leptospire being very thin, their width has to be enhanced to view them in bright field microscopy.

Following techniques were used ----

1. Giemsa staining: ⁽⁵⁾

A smear was prepared and allowed to dry. It was fixed with methanol for 3 minutes and then by a mixture prepared by diluting 0.5 ml Giemsa stain with 10 ml neutral Distilled water was added on the fixed smear and was allowed to stain for 25 minutes. The leptospire stain violet in color.

2. Fontana's Method of staining: ^(5,15)

Solutions prepared:

a. Fixative.

Acetic acid	-	1 ml.
Formalin	-	2 ml.
D/W	-	100 ml.

b. Mordant.

Phenol	-	1 gm.
Tannic acid	-	5 gm.
D/W	-	100 ml.

c. Ammoniated Silver Nitrate.

10 % ammonia was added to 0.5 % solution of Silver nitrate in distilled water until precipitate formed just dissolves. More Silver nitrate was added drop by drop until precipitate returned and did not redissolve.

A smear was made and air-dried, the film was treated 3 times 30 seconds each with fixative. Fixative was washed off with absolute alcohol and allowed to act for 3 minutes. Excess alcohol was drained off and the remainder was burned off till the film was dry. Mordant was poured and heated till steam rose and allowed to act for 30 seconds. The slide was washed with distilled water and dried. Treatment with ammoniated silver nitrate was given with heating till steam rose, and film became brown in colour. The slide was then washed well in distilled water and mounted in Canada balsam.

The spirochetes were stained brownish – black against brownish – yellow background.

The samples that were suspected positive by microscopy were proceeded for further serological investigations and culture isolation.

b. Serological Tests:

The serological tests performed for the confirmatory diagnosis of leptospiral infection in samples were: ---

i. Slide Agglutination Test (SAT).⁽²⁾

To perform this test the serum samples that were positive by microscopy were used.

It is one of the simplest tests still in use; in this test the formalin-killed suspensions of leptospire were mixed with the serum samples and were rotated for 4 minutes. The positive samples showed agglutination which was scaled from 0 – 4.

ii. Microscopic Agglutination Test (MAT).^(2,15)

Microscopic agglutination test is termed as the gold standard method for diagnosis and confirmation of leptospirosis in both Humans and Animals.

The positive serum samples were considered for this test so as to know the antibody titre in the blood of the animal. To perform the test a sterile microtitre plate is taken and the test is performed as follows -----

1. 49 μ l of PBS was added in the wells of the 1st row; except the rows kept for positive and negative control in which 40 μ l PBS was added.
2. 1 μ l test serum was added in the wells with 49 μ l PBS, and 10 μ l positive sample (confirmed) in the well for positive control.
3. 25 μ l PBS was added in all the remaining wells i.e. 2nd 3rd and so on.
4. The dilutions were done by transferring 25 μ l from the 1st row to the second, mixing then to the 3rd, again mixing and so on. The resulting dilutions were 1 : 50, 1 : 100, 1 : 150, so on.
5. 25 μ l of antigen (standard culture of a known strain with at least 100 non clumped organism per microscopic field) was added to all the wells.
6. The plate was gently rotated on a rotator and kept for incubation at 30⁰ C for 2 hours.
7. Then the agglutination was checked microscopically under DFM using a 30 x objective by comparing it with positive and negative control.
8. The maximum dilution of each sample showing 50 % agglutination and 50 % free organisms was considered as the antibody titre.

c. Blood and Urine culture:

The above confirmed blood and urine samples were then cultured in selective medium like ---

1. Leptospira Medium Base.
2. Fletcher's medium.

These media were made selective by adding 5 – Flourouracil and Neomycin Sulphate in them so as to avoid the growth of any present bacterial and fungal contaminants.

6. Culture Isolation and Confirmation:

The organisms grown in the selective media after confirmation with serological diagnosis were then compared with the leptospiral strains of the standard cultures and then confirmed and maintained in the laboratory conditions.

RESULTS AND CONCLUSION:

1. The species wise distribution of the rodents in the study area was determined, it was done separately for the dead and live rodents as follows ----

Dead rodents: -

One hundred twenty (120) dead rodents were collected for leptospirosis survey for which the species wise distribution was as under ---

Species.	Percentage.	No. of Rodents.
<i>Rattus rattus</i>	56.6 %.	68
<i>Rattus norvegicus</i>	15 %.	18
<i>Bandicota bengalensis</i>	23.4 %.	28
<i>Mus musculus</i>	5 %.	06

Live rodents: -

two hundred forty (240) live rodents were studied from the different wards the species wise distribution of these was as under ----

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Species	Percentage.	No. of Rodents.
<i>Rattus rattus</i>	28.4 %	67
<i>Rattus norvegicus</i>	19.3 %	46
<i>Bandicota bengalensis</i>	47.2 %.	112
<i>Bandicota indica</i>	2.5 %.	6
<i>Mus musculus</i>	3.2 %.	8
<i>Suncus cereleus</i>	0.4 %	1

2. Processing of samples from the Dead rodents:

The kidney samples of the dead rodents were processed for microscopic examination and the DFM was performed. One kidney sample of *Rattus norvegicus* from Bhandup area was DFM positive, but was negative by culture isolation method.

3. Processing of samples from the live rodents:

Of the total 240 rats, the urine samples were checked by DFM and the positive samples were processed for culture isolation methods.

Serum samples were examined by DFM and the positive samples were studied using serological methods.

Urine samples:

Two urine samples of the one eighty two samples obtained were positive by DFM and were successfully cultured in leptospira medium base, they were as follows:

Area	Species
D ward	<i>Bandicota bengalensis</i> .
E ward	<i>Bandicota bengalensis</i> .

Blood samples:

Six serum samples were positive by DFM and which were further studied using serological methods, as follows:

SAT:

Four samples gave positive results by SAT and two were negative.

MAT:

The four samples positive for SAT gave positive results for MAT, the antibody titres were as follows:

Area	Species	Titre
G/north	<i>Rattus norvegicus</i>	1 : 80
E ward	<i>Rattus norvegicus</i>	1 : 100
E ward	<i>Rattus rattus</i>	1 : 120
D ward	<i>Rattus norvegicus</i>	1 : 120

Discussion:

Leptospirosis, being one of the most common zoonosis, has always been a great danger to the mankind as well as the animals. the present study was undertaken so as to enable us to estimate the presence of the infection in the rodents, which can be termed as the root source of the infection. Hence the results of this study can further help us in prevention of future outbreaks and to study the causative organism and primary host relationship with respect to their surroundings.

References:-

1. Judith Green – McKenzie. Leptospirosis. www.emedicine.com
2. Coghlan J. D. 1996. Leptospira, Borrelia. Mackei McCartney, 14ed, Churchill Livingstone publication.
3. Faine S. 1991. The Genus Leptospira. The Prokaryotes, II^{ed}, Vol. 4,.
4. <http://www.cehs.siu.edu/fix/medmicro/lepto.htm>
5. Babudieri B. 1961, Laboratory Diagnosis of Leptospirosis. Bull. WHO, 24.
6. Taylor J, and Goyal A. N. 1931, Leptospirosis in Andamans, Ind. Jr. Med. Res. 20.
7. <http://www.medmicro.chapter.35..htm>
8. Jacob John, 1996. Emerging and re-emerging bacterial pathogens in India, Ind. Jr. Med. Res.
9. Ratnam et. al., 1993, Leptospira isolates in Madras city, Ind. Jr. Med. Micro. 17 (4).
10. R. Verma and S. K. Srivastava, 1998. Leptospirosis in animals, prevalence and distribution: Proceedings of the 3rd round table conference, Ranbaxy Science Foundation.
11. <http://www.trustmed.com>
12. <http://www.cdc.gov/nicdod/EID/vol8no12/02-0052.htm>
13. Sushil Shah and Nilesh Shah, Bombay Hosp. Jr.
14. Dr. Hemkar R. R., MRDE, Mumbai.
15. Leptospirosis workshop, 2000. B. J. Medical College, Pune,.