PREVALENCE OF LEPTOSPIROSIS IN ANIMALS IN AND AROUND THRISSUR

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Thesis submitted in partial fulfilment of the requirement for the degree of

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DECLARATION

I hereby declare that the thesis, entitled **"PREVALENCE OF LEPTOSPIROSIS IN ANIMALS IN AND AROUND THRISSUR"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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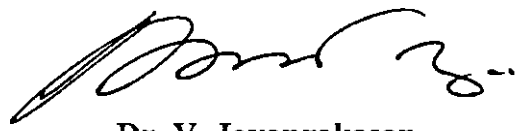
CERTIFICATE

Certified that the thesis entitled **"PREVALENCE OF LEPTOSPIROSIS IN ANIMALS IN AND AROUND THRISSUR"** is a record of research work done independently by **Manju Soman,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.



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We, the undersigned members of the Advisory Committee of **Manju Soman,** a candidate for the degree of Master of Veterinary Science in Microbiology, agree that the thesis entitled **"PREVALENCE OF LEPTOSPIROSIS IN ANIMALS IN AND AROUND THRISSUR"** may be submitted by Manju Soman, in partial fulfilment of the requirement for the degree.

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MANJU SOMAN

Dedicated to My Parents

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Introduction

1. INTRODUCTION

Leptospirosis is a reemerging zoonotic disease of great public health significance, which is caused by antigenically distinct serovars of *Leptospira* affecting all types of warm-blooded vertebrates, in urban and rural contexts and in tropical and temperate climates.

Animals that survive acute infection, which may be feral, peridomestic or domesticated, harbour reservoirs of infectious *Leptospira* in their renal tubules and genital tracts. Human beings are incidental hosts, acquiring infection by contact with infectious material from these carrier or reservoir hosts. Hence prevalence of leptospirosis in human population is generally a reflection of the prevalence in animal population.

Leptospirosis in man and animals have varied manifestations ranging from mild febrile illness to fatal multiorgan dysfunction. In livestock, the disease is a cause of major economic loss due to reduction in milk production, abortion and infertility.

Though varying degrees of endemicity have been reported from different parts of the world, the disease is found to have a predilection for the occupationally exposed groups in the tropical and sub tropical agricultural areas. The incidence of leptospirosis in South India has been reportedly increasing since the last two decades. It is found to occur as epidemics with high mortality lasting for a few weeks during the post monsoon season.

Due to the varied clinical manifestations and complexity in diagnostic techniques, the disease is often not recognized or is erroneously mistaken for other diseases with similar symptoms. Rapid diagnostic aids are hence required for optimal treatment and patient management ,whereas precise identification of the circulating leptospiral serovars is important for epidemiological and public

health surveillance. Information on the role of domestic and peridomestic animals in the maintenance and dissemination of the leptospires in the area would help in adopting suitable control measures.

Isolation of leptospires is the most confirmatory method for demonstrating their presence in the infected animal. But owing to the fastidious nature of this spirochaete, its isolation is restricted to well equipped laboratories only.

Microscopic agglutination test (MAT) is the standard reference method for serodiagnosis of leptospirosis . It helps in the identification of strains of *Leptospira*, assessment of the probable infecting serogroup and confirmation of illness for public health and epidemiological surveillance. The cumbersome nature of MAT and the subjective interpretation of its results makes other genus specific tests like Enzyme Linked Immunosorbant Assay (ELISA) and Passive Haemagglutination Assay (PHA) a better choice as valuable screening tests for leptospirosis.

Passive haemagglutination assay, a comparatively easy test for diagnosis of the disease at an early phase of infection, if conducted in parallel with MAT, may help to distinguish recent infection from a chronic one. Enzyme linked immunosorbant assay for detection of anti leptospiral antibodies has high sensitivity but lack serovar specificity of MAT. A number of modifications of the enzyme immuno assay have been developed using different antigenic preparations, assay protocols and assay platforms.

Post monsoon epidemics of leptospirosis occurring year after year in this part of the country has resulted in heavy loss of human and animal life. Taking this into consideration, the present study was taken up to assess the role of animals in the epidemiology of human leptospirosis, by determining the prevalence of leptospiral antibodies in animals and human beings as well as identifying the common serovars infecting both human and animal population.

Parameters included in this study

1. Detection of leptospiral antibodies in sera collected from apparently healthy as well as infected cattle, dog, pig, rodent and human being in and around Thrissur.

2. Comparison of the efficacy of serological tests – ELISA, MAT and PHA in detecting antibodies against *Leptospira.*

3. Isolation of *Leptospira* from clinical cases of human and canine leptospirosis.

4. Identification of the predominant serogroups of *Leptospira* prevalent in animals and human beings, in and around Thrissur district, by MAT.

5. Isolation of *Leptospira* from rodents captured from premises of cattle farms and households.

Review of Literature

2. REVIEW OF LITERATURE

2.1 HISTORY

Weil in the year 1886 described leptospirosis as an acute febrile illness characterized by jaundice, splenomegaly and nephritis. The term Weil's Disease was first used by Goldschmidt (1887) to designate the condition described by Weil. The spirochaetal aetiology of this disease was proved by Uhlenhuth and Fromme (1915) in Germany, who named it as *Spirochaeta icterogens* and about the same time in Japan by Inada *et al.* (1916) as *Spirochaeta icterohaemorrhagiae.* It was Noguchi (1918) who proposed the name '*Leptospira*' (meaning thin spirals) and created a new genus in the order Spirochaetales following extensive microscopical and cultural studies.

Since then many serovars of *Leptospira* have been isolated from domestic and wild animals (Babudieri, 1958; Twigg *et al.,* 1969), which necessitated the correct identification of these serovars and led to the development of serological classification, based on absorption and cross agglutination (Abdussalam *et al.,* 1972).

2.2 INCIDENCE

The first evidence of leptospiral infection in India was reported by Taylor and Goyle (1931) in human beings, from the Andaman Islands.

Ayyar (1932) reported an outbreak of leptospiral jaundice in Madras hounds and found that it was caused by *L. icterohaemorrhagiae.*

Leptospirosis in cows in USA was reported by Jungherr (1944). Pargaonker (1957), detected leptospiral infection in rats in Hyderabad city through direct microscopic examination and staining techniques.

Adinarayanan *et al.* (1960) studied a disease condition in cattle population in Uttar Pradesh and found that the disease was caused by leptospiral serovars namely *L. sejroe, L. medanensis* and *L. saxkoebing.* A similar condition was reported in cattle in West Bengal by Das and Ghose (1961).

Serological evidence of leptospirosis in pigs in India was first reported by Bhagwat (1964) who carried out a serological study to detect the cause of abortion in a small unit of six pigs in Aurungabad. All the pigs showed suggestive titres to serovar *pomona* without sign of illness.

The first record of leptospirosis in Madhya Pradesh was made in goats by Kharole and Rao (1968). Leptospires were demonstrated in the kidney by dark field microscopy (DFM) and staining techniques. Goats were found to harbour the leptospires without any clinical evidence.

Carlos *et al.* (1971a) reported *L. grippotyphosa* infection in a cat showing febrile and icteric manifestation in Philippines.

Incidence of leptospirosis in breeding bulls was studied by Rameshkumar *et al.* (1990) in Erode district of Tamil Nadu and found that Jersey and Zebu breeding bulls had an incidence of 3.1 per cent and 8.53 per cent respectively, while buffalo breeding bulls showed no incidence of leptospirosis.

Sera collected from sheep during an outbreak of jaundice, abortion and death in five villages in Tirunelveli district of Tamil Nadu showed high agglutination titres by MAT to *L. pomona* (Manickavel *et al.,* 1991).

Ramakrishna and Venkataraman (1994) recorded an incidence of leptospirosis in 16.3 per cent of dairy cattle with symptoms of anorexia, mastitis, abortion and infertility from various places (Nellaikattabomman and North Arcot Ambedkar districts) of Tamil Nadu from January1988 to December1990, with *L. hardjo* being the predominant infecting serovar.

The first report of pulmonary leptospirosis in human beings from India was from the Diglipur tehsil of North Andaman during October to November 1993 in which the most prevalent serovar was *L. grippotyphosa,* followed by *L. canicola* and *L. JEZbratislava* (Sehgal *et al.*, 1995).

Chandrasekaran *et al.* (1995) following a study on the incidence of leptospirosis in Madurai Medical College from1991 to 1992 detected 48 per cent and 54.5 per cent positivity by DFM in the respective years with students and medical staff accounting for more than 50 percent of the leptospiral infections.

Singh *et al.* (1999) in a study on hospitalized cases in Port Blair from September 1996 to August 1997 found that the incidence of human leptospirosis had two peaks, one during July and other during October to November.

An outbreak of human leptospirosis during a rainy season in suburbs of Mumbai was investigated by Bharadwaj *et al.* (2002) and found that serovar *copenhageni* of serogroup *Icterohaemorrhagiae* was responsible for most of the infections.

2.3 PREVALENCE

Stuart (1946), in a serosurvey conducted in dogs in Glasgow, found that about 40 per cent of house dogs were infected with *L. canicola* and about six per cent with *L. icterohaemorrhagiae*. In kennel dogs 28 per cent were infected with *L. icterohaemorrhagiae.*

Murphy *et al.* (1958) reported a prevalence of 16.2 per cent in a serosurvey for leptospirosis conducted in dogs from rural areas in Pennsylvania and neighbouring states. The predominant serogroup was *L. autumnalis* (41 per cent), followed by *L. canicola* (24 per cent) and *L. icterohaemorrhagiae* (12 per cent). Titres against *L. grippotyphosa* (eight per cent) and *L. ballum* (three per cent) were also detected.

Pargaonker (1964) reported leptospiral infection among sheep and goats in Andhra Pradesh. He found that out of the 50 sera samples collected from sheep and goats with history of abortion, 17 had antibodies to serotypes of *L. pomona, L. hyos* and *L. hebdomadis* groups with serum titres ranging from 1: 300 to1:10,000.

Palit and Sharma (1971a) screened 283 sera samples from different species of animals from different parts of India for antibodies to *L. pomona, hebdomadis, autumnalis, icterohaemorrhagiae, canicola* and *poi*. They observed significantly high titres for *L. pomona* in buffalo sera (1:1000 to 1:3000).

Hanson *et al.* (1971) demonstrated the presence of *L. grippotyphosa* infection in sows having clinical signs of leptospirosis, by serology and cultural techniques.

Results of a serological survey of 507 dogs in Manila and environs indicated that 13 per cent of the dogs had significant antibody titres to *Leptospira*. The most common serogroup detected by MAT was *L. bataviae*, followed by *L. javanica* and *L. icterohaemorrhagiae*. The evidence of serology was substantiated by six isolates obtained from 60 selected febrile and icteric dogs (Carlos *et al.*, 1971b).

Rajasekhar and Nanjiah (1971) in a sero-prevalence study on leptospirosis in domestic animals in Mysore state, detected that 4.6 per cent of bovine sera samples contained agglutinins, predominantly to *Leptospira wolffi/borincana/sejroe*, while in pigs, 27.6 per cent were positive, with *L. pomona* being the predominant serovar. Antibodies to *L. pomona* were detected in 23.1 per cent of positive goat sera samples and in 2.2 per cent of sheep sera samples. Agglutinins to *L. canicola* were observed in 10 per cent of canine sera while *L. icterohaemorrhagiae* and *L.wolffi* were the prevalent serovars in horses having a prevalence of 14.3 per cent.

Khera (1972) reported an overall prevalence of leptospirosis in 29.5 per cent of livestock in India, following serological analysis of sera from different species of livestock in 17 states of India. Among this Kerala had the maximum prevalence of 65 per cent. The minimal prevalence of 23.5 per cent was recorded in Punjab.

Serological evidence of leptospirosis in horses was reported by Rajasekhar *et al.* (1977) in Karnataka by detection of antibodies against *L. icterohaemorrhagiae* and *L. wolffi* by MAT.

Tripathy (1977) observed the presence of leptospiral agglutinins in cattle, sheep and goats in Orissa. The prevalence rates were 44.37 per cent, 17.56 per cent and 50 per cent respectively by rapid slide agglutination test using an antigen pool consisting of *L. autumnalis, L. pomona* and *L. wolffi.* He also found that serovar *autumnalis* predominated in all the animals when tested with individual antigens.

Adinarayanan and James (1980) carried out a three year study on leptospirosis in Kerala in which detailed investigation was carried out in porcine, bovine, ovine, caprine, murine and some other wild animal species at Mannuthy and Thumburmuzhy. Twenty eight strains of *Leptospira* were isolated from divergent animal hosts.

Verma (1982) screened 137 sera samples, collected from apparently healthy and clinically infected dogs, for leptospirosis in the state of Punjab. The overall prevalence rate was found to be 34.3 per cent, involving mainly *L. canicola* and *L. icterohaemorrhagiae.*

The significance of leptospiral antibody titres associated with abortion in cattle above eighteen months of age in Southern Queensland was studied by Elder *et al.* (1985), following an investigation spanning four years from January 1975. It was found that MAT titres to serovars *hardjo* and *pomona* were related

to abortions in beef and dairy cattle, with *L. pomona* having a greater influence than *L. hardjo* on the abortions.

Sixty one sera samples from cattle and buffaloe, presented at the Madras Veterinary College with problems of repeat breeding and anoestrum were screened for leptospiral infection by MAT. Twenty six out of the 61 sera revealed titres ranging from 1:20 to 1:320. Three repeat breeding and two anoestrus cattle exhibited titres of 1:80. Antibodies against serovars *autumnalis, hebdomadis, icterohaemorrhagiae, pomona, canicola* and *grippotyphosa* could be detected (Kulasekar *et al.*, 1990).

Batra *et al.* (1990) carried out a serosurvey for detecting leptospiral antibodies in farm animals in Haryana state. Positive titres were seen more in cattle (44 per cent), followed by goats (30.6 per cent) and sheep (29.8 per cent). Thirty five per cent of the healthy and 80 per cent of clinically suspected animals were serologically positive. In cattle and goats *L. wolffi* antibodies predominated where as in sheep *L. pomona* predominated.

Chappel *et al.* (1992) screened pigs slaughtered at three Victorian abattoirs in Australia for leptospirosis by MAT and Immunoglobulin-M enzyme immuno assay (IgM EIA). The study showed that 18 per cent of the pigs were infected or had been infected during their life time.

A serological survey conducted in breeding buffalo herds in Italy from November 1987 to October 1988 using MAT showed 67 per cent positivity. High prevalence of antibodies against serogroups *Saxkoebing* (38 per cent), *Hardjo* (37 per cent), and *Sejroe* (35 per cent) were recorded in all the herds (Ciceroni *et al.*, 1995).

Richardson *et al.* (1995) carried out a serological survey in dairy cows on Prince Edward Islands, Canada, using MAT and detected significant titres to *L. hardjo, pomona* and *bratislava.*

Natarajaseenivasan and Ratnam (1997) carried out a serosurvey during January to February 1995, subsequent to paddy harvest in Punpozhil village in Tamil Nadu. In cattle, prevalence rate was 61.5 per cent with equal distribution to serogroups *Sejroe, Autumnalis* and *Pomona*. Field rats had a prevalence of 52.9 per cent, with *L. pomona* being the predominant serogroup while agricultural workers had a prevalence of 72.5 per cent with *L. autumnalis* being the most prevalent serogroup.

Gangadhar and Rajasekhar (1998) in an attempt to detect the natural reservoir hosts among rodent species, caught 500 rodents belonging to *Rattus rattus wroughton hinton* (248), *R. rattus rufescens* (126), *Rattus bengalensis* (34) and *Bandicota indica* (92) from several human dwelling areas and animal habitats in Karnataka state. Leptospires could be isolated from 89 rodents of which one from *Rattus hinton* was typed as *L. javanica*.

Murhekar *et al.* (1998) detected an overall prevalence rate of 54.2 per cent to *Leptospira* serovars *australis, grippotyphosa, canicola* and *icterohaemorrhagiae* in human beings in the Diglipur tehsil of North Andaman.

Subclinical leptospirosis was detected in cattle, buffaloe and sheep in organized farms in Andra Pradesh by a serological study conducted by Mrunalini and Ramasastry (2000). The total prevalence rate by MAT was 10.58 per cent, with the highest (28.94 per cent) in Adilabad district and lowest in Prakasam district (3.5 per cent). The most common serovars encountered were *L. hardjo* and *L. andamana*.

Sharma *et al.* (2000) studied 50 cases of hepatorenal dysfunction in human beings over a period of two years, in and around Pune. Urine samples were examined by DFM and culture, while sera samples were tested by MAT and microscopic slide agglutination test (MSAT). Positive results were obtained in 88.2 per cent of cases by DFM, 41.1 per cent by culture and 94 per cent by serology.

Biswal *et al.* (2000) found that 60 to 100 per cent of cattle sera collected from different farms in Orissa had antibody titres to leptospiral serovars such as *L. javanica* (26.9per cent), *L. australis* (15.2per cent), *L. autumnalis* (12.8 per cent), *L. pomona* (8.6per cent) and *L. icterohaemorrhagiae* (5.1per cent). Titres to serogroup *Icterohaemorrhagiae* were detected in 50 per cent of the farm workers.

Seroprevalence of leptospirosis in parturient sows in Japan was investigated and it was found that sera samples obtained from the Gunma Prefecture of Japan had significant titres to *L. copenhageni* and *L. canicola* whereas sera samples obtained from Chiba Prefecture were positive to *L. icterohaemorrhagiae* and *L. canicola* (Kazami *et al.*, 2002).

Chaudhry *et al.* (2002) conducted a systematic study on the current status of human leptospirosis in North India from April 2000 to March 2001 and found that highest number of ELISA positive cases, suggesting an epidemic, were seen during the months of August and September 2000.

A serosurvey for leptospirosis was conducted in rice mill workers as well as domestic and peridomestic animals living on the mill premises, in Salem. It was found that mill workers had a prevalence rate of 68.3 per cent while cattle, dogs, cats and rats had prevalence rates of 52.9 per cent, 50.0 per cent, 66.6 per cent and 52.1 per cent respectively by MAT (Natarajaseenivasan *et al.*, 2002).

2.4 EPIDEMIOLOGY

Pargaonker (1957) opined that *Leptospira* which cannot survive outside the carrier host for long, can survive in stagnant waters contaminated by fresh urine, leading to spread of infection.

Sullivan (1974) reported the world wide distribution of leptospirosis and the cosmopolitan nature of serotypes *pomona* and *icterohaemorrhagiae*. He

opined that urinary excretion of leptospires was the most significant factor in the epidemiology of leptospirosis.

The association of human leptospirosis with heavy rainfall has been reported by many workers (Sullivan, 1974; Rathinam *et al.*, 1997; Brandao *et al.*, 1998; Singh *et al.*, 1999; Lomar *et al.*, 2000; Bharadwaj *et al.*, 2002).

Heath and Johnson (1994) reasoned out that because of high concentration of leptospires in urine (10^5 organisms/ml of urine), during the first few weeks of infection, splashing of urine play an important role in transmission.

Natarajaseenivasan and Ratnam (1997) conducted an epidemiological study on leptospirosis in cattle, goat, sheep, rodents (field rats), agricultural workers and people engaged in non agricultural occupation. *Leptospira autumnalis* was found to be the infecting serovar among agricultural workers while serovars *pomona* and *autumnalis* predominated in sheep, goats, rats and cattle. Hence, it was concluded that human infections could have occurred from domestic animals and field rats.

Brandao *et al.* (1998) reported that outbreaks of human leptospirosis in Sao Paulo, a heavily populated metropolitan area in Brazil, occurred as a consequence of severe flooding during monsoon.

Murhekar *et al.* (1998) in a serosurvey conducted in Diglipur in North Andaman found that people who were using stream water for domestic consumption and those with constant contact with dogs were at high risk to *L. grippotyphosa* whereas, farming people in contact with cattle were at risk to *L. australis* and those bathing in ponds were at risk to *L. canicola*.

A clinico-epidemiological study was carried out on hospitalized cases of human leptospirosis in Port Blair by Singh *et al.* (1999) for a period spanning from September 1996 to August 1997. The results showed two separate peaks of the disease, roughly coinciding with paddy sowing and harvesting. It was also

found that majority of patients had exposure to wet or waterlogged soil prior to illness.

Smits *et al.* (2001) reported that people living under poor socio-economic and unhygienic conditions were at particular risk of getting leptospirosis.

In an epidemiological investigation carried out in and around the premises of a rice mill in Salem, it was found that the most predominant serogroups infecting man and animals including rodents were *autumnalis* and *icterohaemorrhagiae*. Following this, *L. icterohaemorrhagiae* could also be isolated from an infected worker. Hence, it was reported that the rice mills which provided abundant food to rodents could be an epidemiological niche for transmission of leptospirosis (Natarajaseenivasan *et al.*, 2002).

A serological study conducted on rodents in Rochefort, France near the Genoulle canal showed that out of the 130 rodents tested, 30.8 per cent were positive, with a predominance for *L. icterohaemorrhagiae,* followed by *L. saxkoebing* and *L. australis*. It was concluded that rodent excreta could have been responsible for the cases of leptospirosis reported in persons who had swum the Genoulle canal (Perra *et al.*, 2002).

Rathinam (2002) reported the emergence of new risk factors for leptospirosis in developed countries such as recreational health hazards associated with water sports and adventure travel.

2.5 CLINICAL SYMPTOMS

2.5.1 Disease in Dogs

Ayyar (1932) in a study conducted on Madras Hounds found that leptospiral infection was characterized by sudden off feed, sanguinous discharge from the nostrils, conjunctivitis, melena and death. Icterus with haemorrhagic symptoms were noticed towards later stages of infection. Vomiting and pyrexia were also recorded in some cases.

Sullivan (1974) reported that classically *L. icterohaemorrhagiae* infection was associated with severe liver involvement, making icterus an outstanding feature, while *L. canicola* infection was manifested mainly by kidney damage with consequent signs of renal failure like uraemia.

Dogs with abortion histories were found positive by MAT, with predominant reaction to serotypes *canicola, pyrogenes* and *ballum* (Jelambi *et al.*, 1976).

Leptospirosis due to *L. bratislava,* characterized by increased thirst, variable appetite, weakness of hindquarters, pyrexia, abdominal pain and jaundice was reported in a German Shepherd dog by Thomas (1980).

Meningeal involvement in leptospirosis in dogs resulting in nervous symptoms was reported by Hungerford (1990).

2.5.2 Disease in Cattle

Sullivan (1974) reported that *L. hardjo* infection in cattle were characterized by flaccid/cold mastitis which lasted for three to four days where as late abortion was the first indication of *L.pomona* infection in adult cattle . Infection in calves by serovar *pomona* was characterized by fever, haemoglobinuria, anorexia and variable degree of icterus.

Kulasekar *et al.* (1990) detected leptospiral antibodies in sera samples from cows and buffaloe with problems of repeat breeding and anoestrum.

2.5.3 Disease in Swine

Ferguson and Power (1956) observed fever, joint stiffness, inappetence and abortion in sows experimentally infected with *Leptospira.*

Cole (1990) observed that leptospirosis in pigs were usually subclinical/ asymptomatic and caused late abortions but occasionally metritis, icterus, anaemia, fever and meningo-encephalitis were also seen .

Kazami *et al.* (2002) reported leptospirosis in parturient sows characterised by premature birth and farrowing of weak piglets.

2.5.4 Disease in Human Beings

Clinical presentation of human leptospirosis was reported to be biphasic with the acute or septicaemic phase lasting about a week, followed by the immune phase characterized by antibody production and excretion of leptospires in urine (Turner, 1967).

Ocular leptospirosis characterized by uveitis, which occurred as a late complication of systemic leptospirosis and which involved one or both eyes with panuveitis had been reported by several authors (Rathinam *et al.*, 1997; Rathinam and Namperumalsamy, 1999).

Singh *et al.* (1999) described two separate syndromes in the clinical course of the disease viz., hepatorenal and pulmonary, with the classical biphasic nature evident only in hepato renal syndrome. The prognosis was reported to be grave in the pulmonary form of the disease.

Leptospirosis in human beings was reported to occur in two clinically recognizable syndromes. The icteric form described in 1886 by Weil was associated with severe hepatic, renal, cardiac and pulmonary malfunctions, with haemodynamic, and neurological alterations. The self limiting anicteric form was characterized by sudden onset of generalized head ache, reticulo-ocular pain, muscle pain , high fever, conjunctival suffusion and aseptic meningitis (Lomar *et al.*, 2000).

2.6 LABORATORY DIAGNOSIS

2.6.1 Immunochemistry of Leptospires

Palit and Gulasekharam (1973) observed that the broadly reactive genus specific erythrocyte sensitising antigen used in PHA, prepared by sodium tauro

cholate treatment and alcohol precipitation, detected IgM antibodies whereas, the heat extracted antigen used in ELISA detected both IgM and IgG antibodies.

Thiermann and Garrett (1983) reported that the hot-phenol-water extracted antigen, used in ELISA had somatic antigenic components, while MAT reactions were mostly based on agglutination of surface antigenic components present on live leptospires.

Mendoza and Prescott (1992) reported that the axial filaments (AF) extracted from *L. interrogans* serovar *canicola,* by cesium chloride density gradient centrifugation of sarcosyl treated whole cells, efficiently substituted sonicated whole cells as genus specific antigen for ELISA.

Yan *et al.* (1999) extracted four different leptospiral antigens from *L. hardjo*. Of this, the outer envelop antigen and the protoplasmic cylinder antigen were protein in nature. Monoclonal antibodies (Mabs) were raised against the protein, carbohydrate and whole cell antigens. It was found that Mabs produced against the lipopolysaccharide (LPS) fractions had agglutinating, leptospiricidal and growth inhibiting properties.

Ribotta *et al.* (2000) prepared a heat extracted antigen from serovar *pomona* which on subjecting to SDS-PAGE and immunoblot, revealed bands around 14.4 KDa region and 19 to 27 KDa region. The antigenic preparations from other serovars showed bands around 14.4 KDA region. The findings of this study supported the observations by Terpstra *et al.* (1985) that this antigen could be prepared from different leptospiral serovars and may contain group specific and possibly type specific fractions, apart from the broadly reactive genus specific fractions. They opined that serogroup/serovar specific leptospiral antigens mostly contained lipopolysaccharide (LPS) while serogroup cross reactive antigens were composed of proteins

2.6.2 Antigen Detection

2.6.2.1 Microscopic Demonstration

2.6.2.1 a Dark Field Microscopy (DFM)

Alexander *et al.* (1957) detected live leptospires in urine of dogs infected with *L. canicola* by DFM.

Rahman and Macis (1979) opined that intact leptospires were necessary to get a positive result by DFM as dead leptospires may be confused with proteinaceous filaments.

Thiermann (1980) recorded that leptospires could be seen under DFM only in urine with pH value higher than 6.5.

Differential centrifugation of blood at 3000rpm followed by15,000 rpm was carried out by different workers to demonstrate leptospires by DFM (Chandrasekaran and Pankajalakshmi, 1997; Chandrasekaran *et al.*, 1998). Centrifugation at 1000 rpm followed by 3000 rpm was carried out by Vijayachari *et al.* (2001a).

Vijayachari *et al.* (2001a) evaluated DFM as a rapid diagnostic aid for leptospirosis and found that DFM had only 40.2 per cent sensitivity and 61.5 per cent specificity and opined that though it was a quick and easy method for early diagnosis, it was unreliable as it gave a high number of false positive and false negative results. They found that at least 1 x 10^5organisms/ml of serum and 5x 10^4 organisms/ml of plasma were required to demonstrate the leptospires under DFM.

2.6.2.1b Staining

Pargaonker (1957) detected leptospires in *kidney* tissues and urine samples of rats by staining impression smears of kidney *tissues* and air dried smears of urine by Fontana's method and tissue sections by Levaditi's method.

Chappel *et al.* (1992) examined pig kidneys histologically by Warthin-Starry staining and immunogold silver staining. They compared the results with culture and DNA hybridization and found all to be highly specific for detection of leptospires.

Kharole and Rao (1968) demonstrated leptospires in goat kidneys by Levaditi's staining of tissue sections.

2.6.2.1c Immunoperoxidase Test (IPT)

Immunoperoxidase test for detection of leptospires in sections of formalin fixed paraffin embedded kidney tissues was carried out by using serovar specific rabbit antileptospiral IgG and peroxidase labeled swine antirabbit IgG. A good correlation was obtained between culture and IPT (Ellis *et al.*, 1983).

An IPT was evaluated for detection of leptospires in blood and urine of experimentally infected bull calves by Koothan *et al.* (1987).

2.6.2.1 d Fluorescent Antibody Test (FAT)

Ellis *et al.* (1982) demonstrated leptospires by direct FAT in liver, lung, kidneys and cotyledons of aborted fetuses.

Bolin *et al.* (1989) compared FAT with bacteriological culture to detect *Leptospira interrogans* serovar *hardjo* in bovine urine, using Fluorescein labelled rabbit anti *hardjo* antibody. It was found that though FAT was considered as a rapid test ,its sensitivity depended on the antigenic integrity of the organism.

2.6.2.2 Serology

Enzyme Linked Immunosorbent Assay (ELISA)

A Biotin/Avidin double antibody sandwich ELISA was developed to detect leptospiral antigens in bovine urine samples, experimentally added with

serovar *hardjo* type *hardjo bovis*. Immunoglobulin G conjugates specific to three antigenic preparations of the above mentioned serovar (Sonicated antigen, Formalinized-heated antigen and Whole cell antigen) were prepared and used in this ELISA. It was found that all the three conjugates could detect serovar *hardjo* types *hardjo prajitno* and *hardjo bovis* with the same specificity and sensitivity (Champagne *et al.*, 1991)

Saengjaruk *et al.* (2002) developed a monoclonal antibody based ELISA for detection of pathogenic leptospires in human urine. Monoclonal antibodies specific to all members of the genus *Leptospira* and those specific to only pathogenic species were used to develop a test that was highly specific, sensitive and rapid.

2.6.2.3 Molecular Diagnosis

Polymerase Chain Reaction (PCR)

Van Eys *et al.* (1989) were the first to develop PCR for detection of *Leptospira* in urine samples of infected cattle.

Gravekamp *et al.* (1993) used two sets of primers for PCR, derived from genomic DNA libraries of *Leptospira* serovars *icterohaemorrhagiae* and *bim* together, which enabled amplification of all presently described pathogenic species of *Leptospira.*

Bal *et al.* (1994) reported the high sensitivity of PCR to detect leptospires in urine of patients in the first week of illness.

Ramadass *et al.* (1997) used PCR to detect leptospires in urine and serum samples of dogs, cattle and human beings and found that this test was highly sensitive when compared to DFM.

Heinemann *et al.* (1999) compared PCR with culture to detect leptospires in bovine semen. Eighty percent of semen samples were positive by PCR, where

as none were culture positive. Among PCR positive bulls, 35 per cent did not have antibodies to *Leptospira* by MAT.

Leptospiral DNA in the aqueous humor of horses with recurrent uveitis was detected by PCR assay by Faber *et al.* (2000) and compared with serologic testing and isolation. Polymerase chain reaction showed 70 per cent sensitivity while serology had 85.7 per cent sensitivity.

Clinical application of PCR assay for detection of leptospires in urine was evaluated by Harkin *et al.* (2003) and found that positive PCR results prior to seroconversion was useful for an early diagnosis.

2.6.2.4 Isolation

Leptospira was first isolated in India in 1931 by Taylor and Goyle. They isolated *L. andamana* and *L. grippotyphosa* from human patients in Andaman Islands.

Murphy *et al.* (1958) reported the isolation of *L. pomona* from the urine of a dog which had MAT titres to *L. autumnalis*.

Leptospires were isolated from urine and kidney of guinea pigs experimentally inoculated with *L. pomona* (Menges and Galton, 1961). Highest number of isolations were recorded in Fletcher's semisolid media with 10 per cent pooled rabbit sera .

Johnson and Rogers (1964) found that 5-Fluorouracil (5-FU), an analogue of uracil which interferes with the normal biosynthesis of DNA, RNA and cell wall of bacteria, had no effect on the growth and morphology of leptospires.

Sullivan and Stallman (1969) isolated a strain of *Leptospira* serovar *hardjo* from an apparently healthy heifer showing intense leptospiruria and an agglutination titre of 1:30,000 to the serotype. They used a medium which contained 0.2 per cent Tryptose – phosphate broth base with thiamine

hydrochloride, Vitamin B12, Nicotinic acid, and rabbit or foetal calf serum at 10 per cent level.

Carlos *et al.* (1971a) reported isolation of *L. grippotyphosa* from the urine of a febrile and icteric feline.

Nervig and Garrett (1979) described the use of diuretic furosemide to obtain urine samples from cattle, which increased the rate of isolation of *Leptospira* due to a decrease in osmolarity, provoked by furosemide.

The first report of leptospiral isolation from animal species in India was made by Adinarayanan and James (1980) who isolated 28 strains of *Leptospira* from pigs, bandicoots, aborted bovine fetuses, sheep, goat, rat and mongoose, in modified Stuart's and Fletcher's semisolid media. The isolates belonged to serogroups A*utumnalis, Hebdomadis, Javanica* and *Tarassovi.*

Kaveri and Upadhye (1981) could isolate *L. icterohaemorrhagiae* from urine of two clinically suspected dogs in EMJH semisolid media with 5-FU and neomycin sulphate.

Ellis *et al.* (1982) diagnosed leptospiral infection in cattle showing abortion, by isolation as well as by fetal serology and immunofluorescence. Isolation was tried in Stuart's, Korthof's, Fletcher's and various modifications of EMJH media(with agar, rabbit sera and 5-FU). Isolation of *Leptospira* belonging to serogroup *hebdomadis* was possible only in media based on EMJH .

Te Brugge and Louw (1985) used standard EMJH semisolid media (0.15 per cent agar) with EMJH enrichment, 0.5mg/ml of 5- FU and two per cent rabbit serum for isolation of *L. interrogans* serovar *hardjo* from bovine urine and found that rabbit sera increased the growth and isolation rates of leptospires.

Te Brugge and Dreyer (1985) reported the isolation of *L. interrogans* serovar *hardjo* from the urine of dairy cattle with history of abortion or giving birth to premature calves in the Onderstepoort area.

An improved selective medium was developed for isolation of leptospires from clinical material by Adler *et al.* (1986) using the albumin- Tween 80 medium with addition of six antibiotics.

De Lange *et al.* (1987) isolated serovar *pomona* from organs of porcine fetuses as well as renal lymph nodes of slaughtered pigs showing chronic nephritis. Homogenized tissue suspended in Sorensen's buffer was used as inoculum in EMJH semisolid media containing 5-FU @ 0.5 mg/ml.

Venugopal *et al.* (1990) isolated *L. autumnalis* from a clinically suspected patient by culturing whole blood in Korthof's and Fletcher's media . They opined that lack of serum in EMJH media might be the reason for failure of isolation of *Leptospira* in that medium.

Anderson *et al.* (1993) used BSK media (Barbour -Stoenner- Kelly medium) to isolate *Leptospira interrogans* serovar *grippotyphosa* from the skin of a dog which was suspected to have Borreliosis.

Gangadhar and Rajasekhar (1998) in an attempt to detect the natural reservoir hosts of *Leptospira*, isolated leptospires from 89 rodents by culturing their kidneys in EMJH semisolid media. One of these was typed as *L. javanica.*

Natarajaseenivasan and Ratnam (2000) isolated leptospires from dead white albino mice, dead wistar rats, house rats, field rats, sheep and ailing human urine samples in EMJH semisolid media with 0.2 per cent agar, two per cent rabbit serum, one per cent BSA, 0.1 per cent sodium pyruvate and 100 µg/ml of 5-fluorouracil (5-FU). Isolates from rodents and sheep were typed as *L. javanica,* while human isolates were identified as *L.autumnalis* and *L.canicola.*

Sehgal *et al.* (2000) and Natarajaseenivasan *et al.* (2002) could isolate *L. icterohaemorrhagiae* from blood of human beings suspected of having leptospirosis.

Leptospira fainei serovar *hurstbridge* was first isolated from two human patients with Weil's syndrome as reported by Petersen *et al.* (2001).

2.6.3 Antibody Detection

2.6.3.1 Agglutination Tests

2.6.3.1a Macroscopic Agglutination Test

Galton *et al.* (1958) standardized a macroscopic slide agglutination test for rapid diagnosis of leptospirosis. A total of 12 leptopiral serotypes ,combined into four pools of three serotypes each were used for antigen preparation.

Rao and Murthy (1983) standardized a macroscopic slide agglutination test (MSAT) using 15 leptospiral serovars combined into five pools of three antigens each and found that results of MAT and MSAT had a good correlation of 89 to 98 per cent.

Srivastava (1990) compared MAT with a slide agglutination test (SAT) and found that percentage concordance between MAT and SAT in human sera was 64.3 per cent, in cattle sera it was 35.3 per cent and in sheep sera it was 60 per cent. He suggested that SAT alone could not be used for diagnosing leptospirosis in cattle and opined that a low MAT titre and a strong SAT reaction could be suggestive of a recent infection.

Brandao *et al.* (1998) evaluated a commercially available SAT for human leptospirosis and compared it with MAT and IgM ELISA. It was found that SAT and ELISA were statistically more sensitive (99 per cent) compared to MAT as initial screening tests. But SAT was comparatively inexpensive and easy when compared to IgM ELISA.

2.6.3.1b Microscopic Agglutination Test (MAT)

The agglutination lysis test, first developed by Martin and Pettit (1918), laid the basis for the present day MAT.

Schuffner and Mochtar (1927) carried out this agglutination lysis test, using live leptospires as antigen, for the serodiagnosis of leptospirosis.

A detailed evaluation of the effects of density of antigen, method of preparation of dilution, strains of *Leptospira*, incubation period and age of culture on the results of agglutination test was made by Stoenner (1955), who found that antigen of low cell content yielded a higher titre when compared to antigen containing heavy concentration of leptospires.

Murphy *et al.* (1958) carried out microscopic agglutination lysis test employing seven leptospires to detect leptospiral agglutinins in canine sera.

It was Turner in the year 1967 who suggested that the term microscopic agglutination lysis was inappropriate, as lysis of the organisms were not taking place as a result of this test. The test later came to be called as the *microscopic agglutination test.*

Though MAT is considered as the International Standard Technique for diagnosis of leptospirosis due to its high specificity, many workers have described it to be less sensitive, laborious and its interpretation as relatively subjective (Champagne *et al.*, 1991; Ribotta *et al.*, 2000).

Cole *et al.* (1973) developed a MAT, which could be performed and read in microtitre plates.

Manickavel *et al.* (1991) carried out MAT in sheep sera suspected of having leptospirosis and detected high agglutination titres to *L. Pomona,* which were in agreement with the autopsy findings in dead animals.

Microscopic agglutination test carried out for serological survey of dogs for leptospirosis by Brihuega *et al.* (1995) detected 58.07 per cent positive cases, with *L. canicola* as the predominant serovar.

Vijayachari *et al.* (2001b) evaluated MAT as a diagnostic tool during acute leptospirosis and determined the cut off titre most suitable for diagnosis in high and low endemic areas. It was concluded that the diagnostic value of MAT during the first week was very low in highly endemic areas. The best cut off was 1:50 in low endemicity areas during first week and 1:100 in second to fourth week, whereas in high endemicity areas, it was 1:200 during second to fourth week.

Vanasco *et al.* (2001) carried out MAT in sera samples of rodents and compared this with IgG ELISA to determine the optimum cut off dilution for MAT. It was found that difference between positive results of ELISA and MAT were significant at 1:50 and non significant at 1:40 dilution, while there was no difference at 1:20. Hence 1:20 was considered as the cut off dilution in rodents in this study.

A titre of 1:20 and above by MAT was considered as positive in rodents by Natarajaseenivasan *et al.* (2002) who detected *L.autumnalis* as the predominant infecting serovar in rodents with a maximum MAT titre of 1:160, in a serosurvey for leptospirosis conducted at Salem.

Levett (2003) used data derived from MAT to infer the identity of infecting leptospiral serovars or sera groups. In animals, the sensitivity of MAT for predicting the infecting serovar was 46.4 per cent (when cut off was 1:100) and 44.4 per cent (when cut off was 1:800) and the specificity was found to be 64.8 per cent. But it was found that serological analysis was of little value for identification of infecting serovars in human beings.

2.6.3.2 Indirect Haemagglutination Assay (IHA)

The foundation for PHA for the serodiagnosis of leptospirosis was laid by Chang and Mc Comb (1954). They extracted a serologically active erythrocyte sensitizing substance (ESS) from five strains of *Leptospira* and proved the genus specific nature of the ESS.

Palit and Sharma (1971b) compared IHA with MAT and complement fixation test (CFT) for detection of leptospiral antibodies in rabbit and calf hyperimmune sera. The IHA using the ethanol-extracted antigen showed a high degree of cross reactivity among the different leptospiral serotypes while MAT showed little or no heterologous reactions.

Sulzer and Jones (1973) reported that IHA detected leptospiral antibodies in human sera much earlier than MAT, unlike in animals. It was also seen that IHA titres declined in the later part of infection while MAT titres remained unchanged.

Palit and Gulasekharam (1973) standardized a passive haemagglutination test using sheep erythrocytes sensitized with a genus specific leptospiral substance extracted from a water *Leptospira* and tested its efficacy against sera from proven cases of human and animal leptospirosis.

Sulzer *et al.* (1975) evaluated the IHA test for diagnosis of human leptospirosis using soluble, alcohol extracted antigen adsorbed on to human O-negative erythrocytes preserved by pyruvic aldehyde fixation. The test showed an overall sensitivity of 92 per cent.

Morris *et al.* (1977) carried out IHA and MAT in experimentally infected cattle sera and found that both tests detected antibodies by seventh day of inoculation, but unlike MAT antibodies, IHA antibodies were not detectable by the 24^{th} week. Indirect haemagglutination assay titres were usually lower than MAT titres, with little correlation between them. Disulphide bond reduction and gel filtration tests indicated IgM to be the major immunoglobulin detected by IHA while MAT detected both IgM and IgG.

Dwarki (1978) carried out a PHA for the detection of leptospiral antibodies, using genus specific antigens of different leptospires.

Nair (1980) detected leptospiral antibodies in immunized animals using MAT and PHA.

An IHA was standardized test by Srivastava *et al.* (1985) to detect leptospiral antibodies in cattle sera, using sonicated antigen coated on to gluteraldehyde fixed sheep erythrocytes. It was found that there was a positive correlation between MAT and IHA titres, especially when IHA titres were between 32 and 64. Antisera showing MAT titres between 3,200 to 6,400 had IHA titres between 64 and 256 and those having MAT titres below 3,200 had IHA titres between 32 and 64.

Levett and Whittington (1998) evaluated a commercially available IHA test for diagnosis of acute leptospirosis. The test was compared with MAT and ELISA for detection of IgG and IgM antibodies. It was found that IHA had 100 per cent sensitivity and it was confirmed for the first time that IHA could detect both IgM and IgG antibodies.

2.6.3.3 Latex Agglutination Test (LAT)

Ramadass *et al.* (1999) standardized a rapid, semi-quantitative LAT using sonicated leptospiral antigen for detection of leptospiral antibodies in man and animals. Even though less sensitive than ELISA, it was found to be a simple, inexpensive and rapid test.

Smits *et al.* (2000) developed a LAT using a heat stable, broadly reactive antigen prepared from serovar *hardjo* coated onto dyed latex particles. The overall sensitivity was found to be 82.3 per cent and specificity 94.3 per cent.

2.6.3.4 Microcapsular Agglutination test (MCAT)

Sehgal *et al.* (1997) evaluated the utility of a commercial MCAT kit in the diagnosis of leptospirosis and found that the overall sensitivity and specificity of the test compared to MAT were 84.7 per cent and 87.0 per cent respectively The

test was found to have higher sensitivity in detecting early infection compared to MAT.

2.6.3.5 Enzyme Linked Immuno Sorbent Assay (ELISA)

Adler *et al.* (1980) developed a solid phase ELISA to detect specific antileptospiral IgM and IgG antibodies in human sera.

Thiermann and Garrett (1983) developed an ELISA using hot-phenol-water extracted antigen and compared this with MAT for detection of antibodies to *Leptospira interrogans* serovars *hardjo* and *pomona* in experimentally infected cattle. It was found that ELISA using the antiglobulin conjugate was more sensitive than anti IgG ELISA or MAT

A genus specific ELISA for the detection of IgM and IgG antibodies in human sera was developed by Terpstra *et al.* (1985). It was found to be more sensitive compared to other genus specific tests like PHA, as it used a broadly reactive heat extracted antigen with at least four antigenic fractions.

Cousins *et al.* (1985) used ELISA to detect IgM and IgG antibody response to *Leptospria interrogans* serovars *pomona*, *hardjo* and *tarassovi* in cattle, following experimental inoculation. Immunoglobulin-M antibody could be detected one week after inoculation and found to persist for three to five weeks, whereas IgG antibodies persisted for 12 weeks.

Mendoza and Prescott (1992) standardized an ELISA for serodiagnosis of leptospirosis in pigs, using the axial filament of *Leptospira interrogans* serovar *canicola* extracted by cesium chloride density gradient centrifugation, and found it to be remarkably specific and sensitive, compared to MAT.

Gussenhoven *et al.* (1997) standardized a dipstick assay using monoclonal antihuman IgM antibody conjugate, for detection of leptospiral antibodies in human sera.

Winslow *et al.* (1997) evaluated a commercial ELISA to detect IgM antibodies for early diagnosis of leptospirosis. It was seen that ELISA had a relative sensitivity of 100 per cent compared to MAT and a positive ELISA result was seen in many cases before the MAT titres reached 1:50.

Sehgal *et al.* (1999) evaluated the lepto dipstick assay among known cases of leptospirosis in Andaman & Nicobar Islands and found that it had a good level of agreement to the standard MAT.

Srivastava and Tiwari (1999) standardized a Dot-ELISA for detection of leptospirosis in goats using the boiled culture supernatants, of a pool of several serovars as well as individual serovars, as antigen. Dot-ELISA was found to be more sensitive due to its heterologous property and less cumbersome nature when compared to MAT.

The exocellular Mannan isolated from the bacteria *Rhodotorula glutinis* were found to have the same repeating units as the antigenic polysaccharides of *Leptospira biflexa patoc* I (Patoc-APS). Matsuo *et al.* (2000) developed an ELISA using this exocellular Mannan (RM) and found that leptospiral antibodies were immuno reactive to much lower concentrations of R M (4 μg/ml) than Patoc -APS (40 μg/ml) and hence could be used as an efficient replacement to Patoc-APS.

Ribotta *et al.* (2000) used a genus specific heat stable antigen, prepared from formalin killed, heat extracted cultures of nine serovars of *Leptospira*, in an ELISA to detect leptospiral antibodies in dogs. The relative sensitivity and specificity to MAT were 100 per cent and 95.6 per cent respectively.

The relative superiority of IgM plus IgG combined conjugate plate ELISA over the standard MAT test in the detection of leptospiral antibodies among several occupationally exposed groups was determined by Natarajaseenivasan and Ratnam (2001).

Vanasco *et al.* (2001) standardized an ELISA for detection of IgG antibodies in rodents, using sonicated cultures of *Leptospira* as antigen and compared this with MAT and culture.

Surujballi and Mallory (2001) developed a competitive ELISA for detection of antibodies to serovar *pomona* in bovine sera, using monoclonal antibodies and evaluated this with MAT positive sera. The specificity and sensitivity was found to be 96.3 per cent and 93.7 per cent respectively.

Flannery *et al.* (2001) evaluated the diagnostic utility of a recombinant *Leptospira* LPS antigen based IgG ELISA and found that it had a sensitivity of 56 per cent in acute phase sera and 94 per cent in convalescent sera.

Lottersberger *et al.* (2002) developed a genus specific IgG ELISA, using sonicated antigen from cultures of serovar *hardjo,* for diagnosis of bovine leptospirosis. The sensitivity and specificity were 99.4 per cent and 96.1 per cent respectively.

2.6.4 Comparison of Diagnostic Tests

The phenomenon of isolation- positive ,serologically negative rodents has been observed repeatedly by many authors (Shotts *et al.*, 1975; Thiermann,1977)

Thiermann (1983) isolated leptospires from kidneys of four slaughtered cows which were found to be seronegative by MAT .

Comparative evaluation of the sensitivity and specificity of MCAT, PHA and MAT was done by Volina *et al.* (1990). It was found that MCAT and PHA could detect leptospiral antibodies by one to three days of infection with maximum titres on days 11 to 15, while maximum MAT titres were detected on days 21 to 25.

Chappel *et al.* (1992) compared MAT and IgM enzyme immuno assay with bacteriological culture. Culture was positive in 61 per cent of known infected pigs. Microscopic agglutination test had the highest sensitivity of 95 per cent, while IgM EIA had 82 per cent sensitivity.

Levett *et al.* (2001) compared IHA test with IgM dipstick Assay, MAT and culture for diagnosis of leptospirosis. Sensitivity and specificity of IgM assay was 98 and 90.6 per cent, whereas for IHA test it was 92.2 per cent and 94.4 per cent respectively. Leptospires could be isolated in 48 per cent of cases.

Bharadwaj *et al.* (2002) compared MAT with IgM ELISA to detect leptospiral antibodies in human sera and found that in acute phase sera, ELISA could detect 36.6 per cent cases compared to 15.9 per cent cases detected by MAT.

Chaudhry *et al.* (2002) investigated leptospirosis in North India by carrying out DFM, isolation trials, IgM ELISA and MAT on sera samples from suspected patients and found 46.2 per cent positivity by IgM ELISA and 65.6 per cent positivity by MAT .Culture and DFM were negative.

Lottersberger *et al.* (2002) compared an IgG ELISA with MAT for detection of bovine leptospiral antibodies and found that ELISA had a relative sensitivity of 99.4 per cent and specificity of 96.1 per cent compared to MAT. Concordance between MAT and ELISA was measured by Kappa test as 0.96.

Harkin *et al.* (2003) compared PCR assay with bacteriological culture and serological testing by MAT, for diagnosis of lepstospirosis in dogs and found that PCR had a sensitivity of 100 per cent and specificity of 88.3 per cent. They reported that serological tests were poor in detecting infection in dogs that were active shedders of *Leptospira.*

Materials and Methods

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Glassware

Screw capped test tubes of Borosil brand and steristoppered test tubes of Riviera brand were used for the study .The glassware were washed using mild soap solution, rinsed in running tap water and then in double glass distilled water, dried and sterilized in hot air oven at 160 °C for one hour.

3.1.2 Collection of Clinical Materials

Clinical materials for the study were collected from various places in and around Thrissur during the period from May 2001 to September 2003.

Blood and urine samples from canines, for serology and isolation, were collected from suspected cases presented at Veterinary hospitals in Mannuthy and Kokkalai. Liver and kidney tissues for isolation were obtained from autopsy cases presented at the Department of Pathology. Bovine blood was collected from farms, hospitals, Artificial Insemination centers attached to Kerala Agricultural University (KAU) and health camps conducted in and around Thrissur. Porcine blood was collected from farms and slaughterhouses of KAU in Mannuthy. Rodents were captured from the premises of cattle farms (Vechur and University Livestock Farm) and households in Mannuthy and Thrissur. Blood from clinically suspected human beings were obtained from Medical College Hospital and Mission Hospital, Thrissur. Specimens for isolation trials, from human beings and rodents, were also obtained from hospitals and households in Kozhikode.

3.1.3 Media

3.1.3.1 *Fletcher's Semisolid Medium*

The medium was prepared by dissolving 0.25g of Fletcher Leptospira Medium Base (Hi Media) in 90 ml of triple glass distilled water. This was

sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure and then cooled to 55-60 °C.

Pooled, haemolysed, heat inactivated rabbit serum was added to the medium base at 10 per cent level, to obtain 100 ml of medium, which was mixed and dispensed in three to five millilitre quantities in tubes provided (3.1.1). The tubes containing media were checked for sterility by incubation at 37°C for 48 h and then stored at 4°C until use.

3.1.3.2 Ellinghausen McCullough Johnson Harris (EMJH) medium

3.1.3.2a *EMJH Albumin Supplement*

The following stock solutions were prepared in g/100 ml of sterile triple glass distilled water and stored at-20°C.

Stock Solutions

Zinc sulphate $7.H_2O$	0.1
Calcium chloride $2H_2O$	1.0
Magnesium chloride $6H_2O$	1.0
Copper sulphate $5H_2O$	0.3
Tween 80	10.0
Vitamin B_{12}	0.02
Ferrous sulphate $7H_2O$(freshly prepared)	0.5
Bovine Serum Albumin fraction V (BSA) (freshly prepared)	20

To 100 ml of the BSA solution, the following quantities of working solutions were added.

Working solutions

Zinc sulphate	2.0 ml
Calcium chloride	2.0 ml
Magnesium chloride	2.0 ml
Ferrous sulphate	20 ml
Copper sulphate	0.2 ml
Vitamin B_{12}	2 ml
Tween 80	25 ml

The pH was adjusted to 7.4 using 1N NaOH and final volume made upto 200 ml using sterile triple glass distilled water and then sterilized by filtration through a 0.2 µm membrane filter.

3.1.3.2b ***EMJH semisolid medium***

The medium was prepared by dissolving 0.23g of the Leptospira Medium Base EMJH (DIFCO) in 90 ml triple distilled water to which bacteriological agar (0.15 per cent) was added and sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure.

The EMJH albumin supplement was added at 10 per cent level to the cooled base medium so as to obtain 100 ml of medium, which was mixed and dispensed in three to five millilitre quantities in the tubes provided (3.1.1). The tubes containing media were checked for sterility by incubation at 37°C for 48 h and then stored at 4°C until use.

3.1.3.2c ***EMJH Liquid Medium***

The EMJH base medium was prepared as described in 3.1.3.2.b and sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure, to which EMJH albumin supplement (3.1.3.2a) was added at 10 per cent level and dispensed in five

millilitre quantities in screw capped test tubes. Sterility was checked by incubation at 37°C for 48 h and stored at 4°C until used.

3.1.3.3 *Fletcher's and EMJH Semisolid Medium with 5- Fluorouracil (5-FU)*

Hundred milligrams of 5-FU was added to five millilitre of sterile triple distilled water. To this 0.1 to 0.2 ml of 0.1N NaOH was added and heated to dissolve. The pH was adjusted to 7.4 to 7.6 using 0.1N NaOH and the volume was made upto 10 ml with sterile triple distilled water. The solution was sterilized by filtration through a 0.2 µm membrane filter and stored at -20°C.

One millilitre of this solution was added to 100 ml of the Fletcher's / EMJH semisolid medium to obtain a final concentration of 100 µg/ml of medium. The medium was dispensed in three to five millilitre quantities in the tubes provided (3.1.1) and were checked for sterility by incubation at 37°C for 48 h and then stored at 4°C until use.

3.1.3.4 Blood Agar

The dehydrated blood agar base (Hi-Media) was prepared as per the manufacturer's instructions, to which sterile sheep blood was added at five per cent level to make blood agar.

3.1.4 Buffers and Reagents

3.1.4.1 Alsever's Solution

Glucose	2.05 g
Sodium citrate	0.8 g
Citric acid	0.055 g
Sodium chloride	0.42 g
Distilled water	100 ml

The solution was sterilized by autoclaving at 115°C for 15 min at 10lbs pressure, cooled and stored at 4°C until use.

3.1.4.2 Phosphate Buffered Saline (PBS) (10x Stock Solution)

Sodium chloride	80 g
Potassium chloride	2 g
Disodium hydrogen phosphate (Na_2HPO_4)	11.33 g
Potassium dihydrogen phosphate (KH_2PO_4)	2 g
Distilled water	1000 ml

pH was adjusted to 7.2 by 1N NaOH and sterilised by autoclaving at 121°C for 15 min at 15 lbs pressure.

3.1.4.3 PBS (1 x Working Solution)

PBS (10 x) (3.1.4.2)	100 ml
Distilled water to make	1000 ml

pH adjusted to 7.2 and stored at 4 °C until use.

3.1.4.4 PBS- Tween- 20 (PBST) – 0.05 per cent

Tween 20	500 µl
PBS 1x (3.1.4.3)	1000 ml

pH adjusted to 7.2 and stored at 4°C .

3.1.4.5 BSA-PBST - 0.5 per cent

BSA	0.5 g
PBST (3.1.4.4)	100 ml

A fresh solution was prepared before use.

3.1.4.6 Citrate buffer, 0.01 M, pH 5.0

0.2 M sodium phosphate dibasic ($Na_2HPO_4.2H_2O$)	25.7 ml
0.1 M citric acid	24.3 ml
Distilled water	50 ml

pH was adjusted to 5.0 and stored at 4°C.

3.1.4.7 Chromogen

O-phenylenediamine dihydrochloride (OPD) obtained from Sigma – Aldrich.

3.1.4.8 Chromogen/Substrate solution

OPD	4 mg
Citrate buffer (3.1.4.6)	10 ml

Just before use, 10 µl of 30 per cent hydrogen peroxide was added to the freshly prepared substrate solution.

3.1.4.9 Stopping Solution (1.25 M Sulphuric Acid)

Concentrated sulphuric acid	34.7 ml
Distilled water to make	500 ml

3.1.4.10 Fontana's Stain

3.1.4.10a Fixative

Glacial acetic acid	1ml
Formalin	2ml
Distilled water to make	100ml

3.1.4.10b Mordant

Phenol	1ml
Tannic acid	5ml
Distilled water to make	100ml

3.1.4.10c Silver Solution

A 0.25 per cent solution of silver nitrate was prepared in distilled water. Few drops of a 10 per cent ammonia solution was added to this slowly until a precipitate was formed which dissolved on shaking. More amount of silver nitrate solution was added to this until the precipitate reappeared and the fluid became opalescent. The reagent was stored at 4°C, in amber coloured bottles, until use.

3.1.5 Hyper Immune Serum

Two young healthy rabbits were maintained for raising hyper immune serum. These were sensitized with five to ten day old cultures of *Leptospira interrogans* serovar *pyrogenes*, grown in EMJH liquid media (3.1.3.2.c) with a concentration of approximately 2x 10^8 leptospires/ml. Five injections of 1ml, 2ml, 4ml, 6ml and 6ml each of the culture were given, through the marginal ear vein, at seven days interval respectively. Test bleeding was done at weekly intervals to determine the level of increase in specific antibody activity in the serum. The animals were subjected to total bleeding, two weeks after the last injection, The antibody titres were detected as 1:512 by PHA and 1:20480 by MAT. Serum was separated, pooled and stored at -20°C until use.

3.1.6 Sheep RBC (SRBC) suspension

Sheep blood, collected in Alsever's solution (3.1.4.1)from healthy sheep maintained at the University sheep and goat farm, was washed thrice by centrifugation in PBS pH 7.2 (3.1.4.3) and resuspended in PBS to yield a 10 per cent suspension, which was stored at 4°C.

3.1.7 Reference strains of *Leptospira*

The following strains of *Leptospira*, representing 10 different serogroups, were procured from National Leptospirosis Reference Centre, Regional Medical Research Centre, Port Blair, Andaman and Nicobar Islands, India and maintained in EMJH / Fletcher's semisolid medium.

	Serogroup	Serovar	Strain
1.	Australis	*australis*	Ballico
2.	Autumnalis	*rachmati*	Rachmati
3.	Canicola	*canicola*	Hond Utrecht IV
4.	Grippotyphosa	*grippotyphosa*	Moskva V
5.	Icterohaemorrhagiae	*icterohaemorrhagiae*	RGA
6.	Pomona	*pomona*	Pomona
7.	Pyrogenes	*pyrogenes*	Salinem
8.	Sejroe	*hardjo*	Hardjoprajitno
9.	Tarassovi	*tarassovi*	Perepeletsin
10.	Semaranga	*patoc*	Patoc I

3.1.8 Antigen for Microscopic Agglutination Test

Reference strains of leptospires were grown in EMJH liquid media (3.1.3.2.c) for five to ten days, to obtain a density of approximately 2 x 10^8 leptospires/ml of medium. The density was determined as per Alexander (1974). A measured drop was examined under DFM using a 22 x 22 mm cover slip. A count of 100 to 200 leptospires per high power field (45 x) for a 10 µl drop of the culture was considered as optimum.

3.1.9 Antigen for Passive Haemagglutination Assay

3.1.9.1 Erythrocyte sensitizing substance (ESS)

Leptospira interrogans serovar *pyrogenes* and *L. biflexa* serovar *patoc* were grown in 100 ml volumes in EMJH liquid media (3.1.3.2.c) for seven to ten days and tested for growth and purity. The ethanol precipitable ESS was obtained from each serovar as per method of Palit and Gulasekharam (1973) with minor modifications.

The liquid culture was dispensed in 10 ml volumes in sterile green-capped vials and centrifuged at 18,000 x g for 45 min. Immediately after centrifugation, the sediments were pooled and suspended in five millilitre PBS (pH 7.2)(3.1.4.3).

An amount of 0.25 g of sodium tauro cholate (Sigma-Aldrich) was added to the five millilitre suspension of leptospires, mixed to dissolve and incubated first at 37°C for two hours and then at 4°C for 16 to 20 h.

Following incubation, the leptospira-bile salt mixture was added to 45 ml of absolute alcohol (Hayman Ltd), mixed thoroughly and kept for flocculation at 4°C for two hours. The precipitate was collected by centrifugation at 200 x g for five minutes and resuspended in five millilitre PBS pH 7.2(3.1.4.3). The resulting faintly turbid solution constituted the leptospiral ESS.

The optimum concentration of ESS required for maximum sensitization of sheep RBC was arrived at by checkerboard titration as described by Palit and Gulasekharam (1973). The optimum dilution (1:2) was the highest dilution of the ESS which when coated on to the SRBCs showed haemagglutination with the highest dilution of the homologous hyper immune serum (3.1.5).

3.1.9.2 Sensitized SRBC

To one millilitre of the 1:2 diluted suspension of ESS, 0.1 ml of 10 per cent SRBC (3.1.6) was added and the mixture was allowed to react in a 37°C water bath for one hour with intermittent shaking. The sensitized cells were washed thrice by

centrifugation in PBS and resuspended in one millilitre of the same buffer. This constituted the antigen for PHA.

3.1.10 Antigen for Enzyme Linked Immunosorbent Assay

The antigen was prepared as per Terpstra *et al.* (1985). *Leptospira biflexa* serovar *patoc* was grown in 100 ml volumes in EMJH liquid media (3.1.3.2.c) for 10 to 12 days and the number of organisms adjusted to approximately 10^9 leptospires/ml of medium by microscopic counting. The culture was killed with formalin (final concentration of 0.5 per cent v/v) and then heated in a boiling water bath for 30 min, with intermittent shaking. After cooling to room temperature, these were dispensed into green- capped vials in 10 ml quantities and centrifuged at 10,000 x g for 30 min. The supernatant obtained constituted the heat-extracted antigen.

3.1.11 Conjugates

Anti-immunoglobulin G horse radish peroxidase (Anti-IgG HRP) conjugates against dog, cattle and human beings were obtained from Bangalore Genei.

3.2 METHODS

3.2.1 Isolation

3.2.1.1 Clinical cases of canine/human leptospirosis

Bed side inoculation of one or two drops of whole blood, collected from suspected cases of human and canine leptospirosis, was done in Fletcher's (3.1.3.1)/EMJH (3.1.3.2b) semisolid media. The inoculum was mixed thoroughly with the medium and about three to four drops were transferred to a second tube and from that to a third one.

Urine collected aseptically by catheterization from clinically suspected convalescent dogs were inoculated (one or two drops) into Fletcher's and / EMJH semisolid media with 5-FU (3.1.3.3), mixed thoroughly and diluted serially as described before.

Portions of liver and kidney cortex of autopsied cases of clinically suspected dogs were collected by puncturing the organs with finely drawn Pasteur pipettes. These were inoculated into Fletcher's semisolid media (3.1.3.1) with aseptic precautions, mixed properly and diluted serially as described above.

3.2.1.2 Rodents

Live rodents captured were anaesthetized with chloroform and opened with aseptic precautions. Tiny portions of kidney were collected by puncturing the cortex using finely drawn out Pasteur pipettes and urine was collected from the bladder using sterile syringes. These were inoculated into Fletcher's semisolid media (3.1.3.1) , mixed thoroughly and serially diluted as in 3.2.1.1.

All the inoculated tubes were incubated at 37°C for 18 to 24 h and then at 28 to 30°C for a period of two to three months. All the tubes were examined at weekly intervals for the presence of leptospires by Dark Field Microscopy (DFM). Those showing growth were sub cultured in fresh media and the contaminated tubes were discarded.

3.2.2 Serology

3.2.2.1 Collection of Sera Samples

Blood samples from 121 dogs, 100 cattle, 84 pigs, 42 rodents and 154 human beings were collected from various places in and around Thrissur. Sera were separated and stored at -20°C until use.

3.2.2.2 Microscopic Agglutination Test

The MAT was carried out as per Faine (1982) with minor modifications.

A 1:10 dilution of the test serum was prepared by mixing 10 µl of the serum with 90 µl of PBS (pH 7.2)(3.1.4.3).

Ten microlitres of the 1 :10 diluted serum was serially diluted two fold times in PBS (3.1.4.3), to provide dilutions of 1 : 20 to 1 : 20480, in 96 well U

bottom microtitre plates. To 10 µl each of the serum dilution, 10 µl of live antigen (3.1.8) was added. Appropriate antigen controls were set with 10 µl PBS and 10 µl of antigen and the plates were incubated at room temperature for 30min to one hour.

The result was read by examining a drop from each well under high power of DFM for agglutination of leptospires. The end point was recorded as the highest dilution of the serum showing 50 per cent agglutination.

3.2.2.3 Passive Haemagglutination Assay

The PHA was carried out as per the procedure quoted by Morris *et al.* (1977) with minor modifications.

Serial two fold dilutions of the test serum in PBS (pH 7.2)(3.1.4.3) were taken in 20 µl quantities in a U bottomed microtitre plate to provide dilutions of 1:8 to 1:4090. Five microlitres of the sensitized SRBC (3.1.9.2) were added to each well and the plates were gently tapped and incubated first at 37°C for one hour and then at room temperature for 16 to 20 h. Two control wells were included in the test, one positive control with 20 µl of diluted positive serum (1:8) and five microlitre of sensitized SRBC (3.1.9.2) and one RBC control with 20 µl of PBS and five microlitre of sensitized SRBC (3.1.9.2).

The end point was noted as the highest dilution of serum showing haemagglutination.

3.2.2.4 Enzyme Linked Immunosorbent Assay

Indirect ELISA was performed as per Terpstra *et al.* (1985), with minor modifications.

The optimum concentration of undiluted coating antigen, optimum dilution of antihuman, antidog and antibovine IgG HRP conjugates (1:10,000) and optimum test sera dilutions (1: 40 for canine and human sera, 1: 80 for bovine sera) were arrived at by preliminary checkerboard titration.

3.2.2.4.a ELISA for human sera

Hundred microlitre of the undiluted heat extracted antigen (3.1.10) was pipetted into all wells of the Tarson's 96 well flat-bottomed microtitre plates and left to evaporate at room temperature for three days. The plates coated this way were stored in a dry place at room temperature until use.

Before use, the plate was washed four times with PBST (3.1.4.4), allowing the fluid to remain in the wells for one minute during the last washing. The plate was tapped dry onto a lint free absorbent towel.

Hundred microlitres each of the human sera diluted to 1:40 in BSA-PBST (3.1.4.5) were added to the wells in duplicates. Positive and negative controls and a conjugate control without any serum was also set and the plate was incubated at 30°C for one hour.

After one hour, the plate was washed and dried as described before and each well was charged with 100 µl of 1:10,000 antihuman IgG HRP conjugate diluted in BSA-PBST (3.1.4.5) and incubated at 30°C for one hour.

The contents were discarded following incubation and the plate was washed and dried as before.

Hundred microlitres of freshly prepared chromogen/substrate solution (3.1.4.8) was added to all wells including the controls. The plate was incubated in dark at 30°C for 30 min.

The reaction was stopped by adding 100 µl of 1.25 M sulphuric acid (3.1.4.9) to each well.

The optical density (OD) was read at 492 nm in a Multiskan Ascent ELISA reader and data interpreted as per Ribotta *et al.* (2000). The arithmetic mean of the OD values of a few negative sera samples (ten in the present study) and their

standard deviation (SD) were calculated. The cut off value for the interpretation of positive and negative samples was calculated as arithmetic mean plus 4 SD.

Dog sera were screened in the same manner as human sera, whereas for bovine sera, the optimum dilution of test sera used were increased to 1:80.

3.2.3 Statistical Analysis

The results obtained from the three tests were analyzed for percentage agreement and relative sensitivity and specificity by kappa statistics as described by Raj *et al.* (1998).

3.2.4 Serotyping of Isolates

Out of the field sera samples subjected to MAT, the ones presenting the highest titre with the individual reference serovars were employed to define tentatively the serogroup of the *Leptospira* local isolates.

Microscopic agglutination test was performed, with local isolates as antigen and known positive sera samples as antibody, as per the procedure cited in 3.2.2.2.

The titres expressed by both tests were compared to arrive at the serogroup identity of the isolate

3.2.5 Fontana's Staining of Leptospiral Isolates

Fontana's staining was done as per the method described by Davies (1955) with minor modifications.

A drop of the liquid culture was placed on a clean and dry glass slide, and allowed to dry in a slanding position, so that a thin film of the culture was formed on the glass slide .

This slide was first treated with the fixative (3.1.4.10a) for one and half minutes. It was then rinsed in absolute alcohol for three minutes and dried thoroughly with heat. Next the mordant (3.1.4.10b) was applied on to the slide and heated from below until steam arised and it was allowed to react for 30 sec. The

slide was then washed in distilled water, dried and treated with the silver nitrate solution (3.1.4.10c), and heated from below, for 30 sec. The stain was poured off and the slide was washed in distilled water, dried and mounted in DPX mountant.

3.2.6 Sterility test for the cultures of leptospiral isolates

A loopful of the leptospiral culture was streaked on to blood agar and incubated at 37°C for three days and checked for any bacterial growth.

Results

RESULTS

4.1 ISOLATION

Isolation trials were done mostly in Fletcher's semisolid media with 10 per cent rabbit sera (FSMR) .In addition to this, EMJH semisolid media with enrichment and Fletcher's / EMJH semisolid media with 5-FU were also used. Primary isolation of leptospires were possible only in Fletcher's media (FSMR).

Media containing 5-FU could inhibit the growth of contaminants present in the inocula, while supporting the growth of leptospires.

Culture media (without 5-FU) that supported growth of leptospires along with bacterial contaminants, could be purified, and obtained as pure culture by culturing serially diluted inocula in similar media (without 5-FU), failing which, repeated subcultures in media containing 5-FU could purify the culture.

In some cases, contaminating bacteria were able to grow along with the leptospires in the 5-FU media. These could be separated by filtration through 0.2 μm cellulose nitrate syringe filters.

Leptospires could be isolated in media taken in screw capped test tubes as well as sterristoppered test tubes. But cultures of *Leptospira* could not be maintained for long periods (>2months) in sterristopped tubes due to loss of moisture by evaporation.

Initial incubation of the inoculated tubes at 37°C for 18 h was helpful in the present procedure adopted for isolation of leptospires from biomaterials. This enabled to detect bacterial contamination by the second day of incubation. But fungal contaminants when present could be detected only by the sixth or seventh day of incubation.

4.1.1 Canine and Human Leptospirosis

Leptospira could not be isolated from any of the 20 canine samples (15 blood samples, three autopsy specimens and two urine samples) subjected to isolation trials. But in one case, Fletcher's media (FSMR) inoculated with kidney and liver tissues of an autopsied German Shepherd Dog showed the presence of a distinct white subsurface ring on the eighth day of incubation. Though few leptospires were detected on DFM, attempts to maintain the culture by subculture were unsuccessful.

Out of the 30 human blood samples subjected to isolation trials, *Leptospira* could be isolated from one human patient with severe hepatorenal complications, admitted at Medical College, Kozhikode, following bedside inoculation of whole blood in Fletcher's semisolid media (FSMR). Live leptospires were detected by DFM on the 18^{th} day of incubation. The isolate was designated as No.289.

4.1.2 Rodents

Of the 42 rodents captured, kidney tissues (35) and urine samples (four) from 35 rodents (11 bandicoots and 24 rats) were subjected to isolation trials. Leptospires could be isolated from five of them, in Fletcher's semisolid media (FSMR). Of these five isolates, three were from bandicoots caught from households at Thrissur and Kozhikode and from the premises of Vechur livestock farm at Mannuthy. These were designated as BT, M64 and R6 respectively. The other two isolates were from rats caught at Mannuthy and Kozhikode and these were designated as R13 and M63.

Isolates BT and M63 were detected following 18 days of incubation while isolates R6, M64 and R13 were detected following 60, eight and 34 days of incubation respectively. Distinct subsurface opacities were evident in all isolates by the above mentioned incubation periods.

All the isolates of *Leptospira* were checked for their characteristic motility by DFM and staining properties by Fontana's staining technique [Plate 1(A)]. When streaked on blood agar, the isolates yielded no growth even after three days of incubation.

Except M63, all the isolates could be repeatedly sub cultured in Fletcher's semisolid media and maintained at 30 °C in the BOD incubator.

4.2 SEROLOGY

4.2.1 Microscopic Agglutination Test

Reference serovars of *Leptospira* could be maintained in Fletcher's semisolid media by routine subculture.

All the leptospiral serovars except serovar *hardjo* of serogroup *Sejroe* grew to the required concentration, for conducting MAT (approximately 2x 10^8 leptospires/ml), in EMJH liquid media by the fifth day of incubation. Serovar *hardjo* reached optimum concentration by seven to ten days of incubation only.

Serovar *rachmati* of serogroup *Autumnalis* could be included in the screening of 43 bovine sera samples only, as this serovar subsequently became irrecoverably contaminated with other bacteria. Out of the 43 bovine sera samples, four were positive for serogroup *Autumnalis*.

All serovars of *Leptospira,* when suspended in PBS, were actively motile and uniformly distributed without any clumps during the pre and post incubation periods of MAT.

MAT was done for a total of 121 canine, 100 bovine, 84 porcine, 42 rodent and 154 human sera samples [Plate 1(B)].

A titre of 1:80 or above was considered positive for dog, cattle, pig and human sera while a titre of 1:20 and above was considered as positive for rodent sera. The serogroup reacting at the highest titre was presumed to be the infecting one. Sera samples showing same agglutination titres to more than one serogroup (excluding serogroup *Patoc)* were considered as mixed equals.

The results of MAT are summarized in Tables 1 and 2.

Species wise seroprevalence of leptospirosis by MAT is represented diagrammatically in Figure. 1

4.2.1.1 Dog

Out of the 121 sera samples tested, 44 (36.36 per cent) were positive for antibodies to *Leptospira*. Of these 44 positive samples, 17 (38.63 per cent) were positive to *L. pomona*, 16 (36.36 per cent) were positive to *L. australis* and two were mixed equals. Of the two mixed equals, one showed equal titres to serogroups *Canicola* and *Australis* while the other reacted to serogroups *Grippotyphosa*, *Australis* and *Pomona*. The agglutination titres in positive sera ranged from 1:80 to 1:5120 and the highest titres were observed against serogroups *Pomona* and *Australis*.

4.2.1.2 Cattle

Forty seven (47 per cent) of the 100 sera samples tested were positive for antibodies to *Leptospira*. Out of the positive samples, 20 (42.55 per cent) were positive to serovar *hardjo* of serogroup *Sejroe*, nine (19.14 per cent) were positive to serogroup *Patoc* and six (12.76 per cent) were positive to serogroup *Pomona*. Agglutination titres ranged from 1:80 to 1:20480 with highest titres detected against serogroup *Sejroe*.

4.2.1.3 Pig

Out of 84 sera samples tested, 20 (23.80 per cent) were found positive for antibodies to *Leptospira*. Agglutinins to *L. pomona* were detected in eight (40 per cent) of the 20 positive sera samples while agglutinins to *L. grippotyphosa* were detected in six (30 per cent) of the positive samples. Agglutination titres ranged from 1:80 to 1:640 with the highest titre recorded against serogroup *Pomona*.

4.2.1.4 Rodent

Of the 42 samples tested, nine (21.42 per cent) were positive for leptospiral antibodies. Four (44.4 per cent) of the positive sera samples had antibody titres to *L. pomona* while antibody titres to *L. icterohaemorrhagiae* and *L. australis* were detected in two (22.22 per cent) sera samples each. Highest agglutination titre of 1:20480 was recorded against serogroup *Pomona* whereas lowest titre of 1:20 was recorded against serogroups *Australis* and *Canicola.*

4.2.1.5 Human

Of the 154 sera samples tested, 84 (54.54 per cent) were positive for antibodies to *Leptospira.* Antibody titres to serogroup *Australis* were detected in 36 (42.85 per cent) of the positive sera samples whereas antibody titres to serogroup *Pomona* were detected in 24 (28.57 per cent) sera samples. Two of the sera samples were mixed equals, reacting to serogroups *Icterohaemorrhagiae* and *Canicola.* The highest agglutination titres were recorded against serogroups *Pomona* and *Australis* (1:20480).

4.2.2 Passive haemagglutination Assay (PHA)

A titre of 1:32 and above was considered as positive for dog, cattle, pig and human sera while for rodents the cut off titre was taken as 1:16.

The sensitized SRBCs used as antigen in the test could be stored at 4°C for a maximum of five days without any change in its antigenicity following which its quality deteriorated and resulted in aberrant test results.

The erythrocyte sensitizing substance (ESS) which had been kept at 4°C for more than five days, when used for sensitization of SRBC, resulted in the lysis of SRBC.

Gluteraldehyde fixed, tanned and sensitized SRBC gave aberrant test results and hence were not used in the test proper.

PHA was done for a total of 121 canine, 100 bovine, 84 pig, 42 rat and 154 human sera samples [Plate 2(A)].

Species wise seroprevalence of leptospirosis by PHA is represented diagrammatically in Figure. 2

Sixty one (50.41 per cent) of the 121 sera samples tested were found positive in dogs with the highest titre recorded as 1:512. In cattle, of the 100 samples tested, twenty three (23 per cent) were positive with the maximum titre recorded as 1:128.

Out of the 84 sera samples tested in pigs, 16 (19.04 per cent) were positive for leptospiral antibodies while in rodents, 11 (26.19 per cent) of the 42 samples tested were positive for antibodies to *Leptospira* .The maximum PHA titres recorded in both these species were 1:32. Sixty six (42.85 per cent) of the 154 human sera samples tested were positive for leptospiral antibodies with highest titre recorded as 1:512.

4.2.3 Indirect ELISA

Indirect ELISA was done in microtitre plates coated with the heat extracted antigen. It was seen that this antigen could be stored for more than two weeks at 4°C without any deterioration in its antigenicity. But once coated on to the microtitre plates, it could only be stored for a maximum period of one week, at room temperature, following which it gave aberrant test results.

ELISA was done on 104 of the total 121 canine sera samples, 74 of the 100 bovine sera samples and all of the 154 human sera samples collected [Plate 2(B)].

Species wise seroprevalence of leptospirosis by PHA is represented diagrammatically in Figure. 2

Sera from dogs, cattle and human beings showing OD values above 1.260, 1.264 and 1.240 respectively were taken as positive.

Fifty six of the 104 sera samples (53.84 per cent) tested positive in dogs, 48 of the 74 sera samples (64.86 per cent) tested positive in cattle and 96 of the 154 sera samples (62.33 per cent) tested positive in human beings.

4.2.4 Statistical Analysis

The results of the three tests were analyzed by use of kappa (κ) statistics.

4.2.4.1 Comparison of Serological Tests

Percentage of agreement between MAT, PHA and ELISA showed Kappa values above 0.9 ($\kappa>0.9$) for all the species tested in this study.

Comparison of the results of the three tests is shown in Table 3.

A total of 332 sera samples comprising of 104 canine, 74 bovine and 154 human sera were subjected to all the three serological tests.

Among these 98 were tested as positive and 112 were tested as negative by all the three tests.

Leptospiral antibodies were detected by ELISA in 38 sera samples which were negative by MAT and PHA, whereas PHA could detect antibodies in 12 sera samples which were negative by the other two tests. But none of the sera samples, which were negative by ELISA and PHA, were positive by MAT.

Eight of the sera samples, which tested negative by ELISA, gave positive results by MAT and PHA while 14 MAT negative sera samples were tested as positive by ELISA and PHA.

ELISA and MAT detected leptospiral antibodies in 50 sera samples which were negative by PHA.

4.2.4.2 Sensitivity and Specificity of the Three Tests

The relative sensitivities and specificities of the three tests are given in tables 4, 5 and 6.

Keeping MAT as the gold standard test, the relative sensitivity and specificity of ELISA were calculated as 0.9487 and 0.7045 while that of PHA were calculated as 0.6794 and 0.8523 respectively.

The relative sensitivities and specificities of these tests in each of the five species tested is given in tables 7, 8 and 9.

4.3 SEROTYPING OF LEPTOSPIRAL ISOLATES

The results of serotyping of isolates is given in Tables 10 and 11.

Of the five isolates inoculated in the EMJH liquid media, only four (M.64, R13, BT and 289) grew to the required concentration for conducting MAT.

These were tested for agglutination against the known MAT positive sera samples 293,121, 576, R9 and TB12, having maximum agglutination titres of 1:20480, 1:20480, 1:5120, 1:2560 and 1:20480 against serogroups *Australis, Pomona, Grippotyphosa* and *Sejroe* respectively.

On carrying out the test, it was found that sera Nos.121, 293 and 576 had agglutination titres of 1:2560,1:640 and1:640 , respectively to isolate No. M.64.

Sera No.293 and 576 had agglutination titres of 1: 5120 and 1:320 respectively to isolate No. R13.

Sera Nos. 121, 293 and 576 had highest titres of 1:10240 ,1: 5120 and 1:640 respectively to isolate No. BT.

Sera No. 121 and 293 had low agglutination titres of 1:1280 and 1:320 to isolate No. 289. Also 289 failed to show agglutination in sera No. 576.

Table 1. Results of MAT – species wise distribution of antibodies to serogroups of *Leptospira*

	Australis	*Autumnalis*	*Canicola*	*Grippo-typhosa*	*Ictero-haemorrhagiae*	*Pyrogenes*	*Sejroe*	*Pomona*	*Tarassovi*	*Patoc*	Mixed equals	Total
Dog	16		3	1	3			17		2	2	44
Cattle	2	4		4			20	6	2	9		47
Pig			4	6	2			8				20
Rat	2		1		2			4				9
Human	36		2	4	4	4		24		8	2	84

Table 2. MAT titres to different leptospiral serogroups in dog, cattle, pig, rodent and human sera

MAT titres to different leptospiral serogroups in dog

Serum No.	*Australis*	*Autumnalis*	*Canicola*	*Grippotyphosa*	*Icterohaemorrhagiae*	*Pyrogenes*	*Sejroe*	*Pomona*	*Tarassovi*	*Patoc*
1.			1*							
2.								5*		4
3.				1	3*			1		1
4.								1*		1
5.	1*							1		1
6.	1		1	1				1		1
7.	6*			2				4		
8.								3*		
9.	6*									
10.	2*									
11.	7*		1	3	2			3		3
12.								3*		2
13.								2*		2
14.	4*									2
15.	2*									
16.				4				7*		4
17.								2		3*
18.	1		1							
19.								5*		4
20.			1*							
21.								1*		1
22.				1	3*					
23.	1		1	1				2*		1
24.				4				7*		4
25.								3*		
26.	2*							1		1
27.	2*									
28.	6*									
29.	4*									2
30.								3*		3
31.	7*		1	3	2			3		2
32.	2*									
33.	6*			2				4		
34.								2		3*
35.								2*		
36.	1		2*							
37.	3*									
38.								2*		1
39.				6*				2		2
40.								2*		2
41.	2*			1				1		1
42.	1							2*		1
43.	3							4*		
44.	1		1		2*					
	16		3	1	3			17		2

Mixed equals –*Canicola* and *Australis* - 1

Grippotyphosa, Australis and *Pomona* - 1

* The serogroup reacting at highest titre, presumed to be the infecting one

(Contd.)

Table 2 (Contd.)

MAT titres to different leptospiral serogroups in cattle

Serum No.	*Australis*	*Autum-Nails*	*Cani-cola*	*Grippo-typhosa*	*Ictero-haemorr-hagiae*	*Pyro-genes*	*Sejroe*	*Pomona*	*Tara-ssovi*	*Patoc*
1.				1				3		4*
2.								3*		
3.										3*
4.								1*		
5.									1*	
6.	2*									
7.							2*	1		
8.							2*			
9.				1				7*		1
10.							5*			
11.										1*
12.										1*
13.				2*						
14.								3*		
15.		1	1				6*			
16.							1*			1
17.		4*						1		
18.				1*						
19.		1					5*			
20.							6*			
21.		2*								1
22.							9*			1
23.			1	1			8*			
24.										1*
25.							2*			
26.										1*
27.				1				3		4*
28.							1*	1		
29.				1				7*		
30.				2*						
31.										1*
32.		1					9*			
33.								3*		
34.	1	1	1				3*			
35.		1	1				6*			
36.							1*			1
37.		4*						1		
38.		1					5*			
39.				1*						
40.							9*			1
41.	2*							1		
42.							6*			
43.										1*
44.			1	1			8*			
45.		1*								
46.							2*			
47.				1					2*	
	2	4		4			20	6	2	9

(Contd.)

Table 2 (Contd.)

MAT titres to different leptospiral serogroups in pig

Serum No.	*Australis*	*Autumnalis*	*Canicola*	*Grippotyphosa*	*Icterohaemorrhagiae*	*Pyrogenes*	*Sejroe*	*Pomona*	*Tarassovi*	*Patoc*
1.			1*							
2.				1*						
3.					1*					
4.				1*						
5.				1*						
6.			1*							
7.	2			1				4*		3
8.								4*		3
9.								1*		
10.								1*		
11.				1				4*		
12.			1*							
13.					1*					
14.			1*	1						
15.				1*				1		
16.								1*		
17.								1*		
18.				1*						
19.								4*		3
20.				1*						
			4	6	2			8		

MAT titres to different leptospiral serogroups in rat

Serum No.	*Australis*	*Autumnalis*	*Canicola*	*Grippotyphosa*	*Icterohaemorrhagiae*	*Pyrogenes*	*Sejroe*	*Pomona*	*Tarassovi*	*Patoc*
1.	-2*									
2.								9*		8
3.	2							5*		
4.					1*					
5.								1*		
6.	1							9*		6
7.					-1*					
8.			-2*							
9.	1*									
	2		1		2			4		

Serum No. 2 - Bandicoot (BT)
Serum No. 5 - Bandicoot (R6)

(Contd.)

Table 2 (Contd.)

MAT titres to different leptospiral serogroups in human

Serum No.	*Australis*	*Autumnalis*	*Canicola*	*Grippotyphosa*	*Icterohaemorrhagiae*	*Pyrogenes*	*Sejroe*	*Pomona*	*Tarassovi*	*Patoc*
1.	2							7*	2	6
2.	4*							2		3
3.	4					2		6*	2	5
4.	4			4				4		5*
5.	4							8*		6
6.	1*									
7.	3							5*	1	4
8.	4*							1		
9.			1*							
10.	2			8	6			9*		
11.	1		1			1		4*	1	3
12.	1					2*		1		
13.	2*							1		1
14.								3*		
15.				1				3*		2
16.	5				6			7*		6
17.	1*									
18.	2*									
19.	1*									
20.					1*					
21.						4*				3
22.										1*
23.			1		1					
24.	1*									
25.	6*			3				3		5
26.	1*									
27.	9*		5		3			6		5
28.	2*									
29.	3							4*		
30.					1*					
31.	2*									
32.	4					2		6*	2	5
33.					1*					
34.	4							8*		6
35.	1*									
36.	3							5*	1	4
37.			1		1					
38.	9*		5		2			6		6
39.			1*							
40.	1		1					4*	1	3
41.	1*									
42.					1*					
43.	9*							5	2	5
44.	2*							1		
45.			1							5*
46.				1				3*		2
47.				1				2*		
48.			1	1						5*
49.	7*							6		6
50.	8*					1		6		7
51.								1		2*

(Contd.)

Table 2 (Contd.)

Serum No.	*Australis*	*Autumnalis*	*Canicola*	*Grippotyphosa*	*Icterohaemorrhagiae*	*Pyrogenes*	*Sejroe*	*Pomona*	*Tarassovi*	*Patoc*
52.	9*							5	2	5
53.	2			4*				2		2
54.	1*									
55.	2*			1				1		2
56.				4*		1		3		4
57.	3		2			3		5*	3	2
58.	1*									
59.	4*							3		3
60.	1*									
61.	1					4*				3
62.	4*		1					2		3
63.				2				5*		
64.	4			4				4		5*
65.	7*							5		5
66.	6*			3				3		5
67.	1*									
68.	8*					2		6		7
69.	4*									2
70.								1		2*
71.	2			8	7			9*		
72.	2			4*				2		2
73.	2*									
74.	1					2*		1		
75.	2*			1				1		2
76.								3*		
77.				4*		1		3		4
78.	3							4*		
79.	3		2			3		5*	3	2
80.	5				6-			7*		6
81.	4*							3		3
82.										1*
83.	2							6*	2	6
84.	2*									1
	36		2	4	4	4		24		8

Mixed equals – *Icterohaemorrhagea* and *Canicola* - 2

-2 - 1:20
-1 - 1:40
1 - 1:80
2 - 1:160
3 - 1:320
4 - 1:640
5 - 1:1280
6 - 1:2560
7 - 1:5120
8 - 1:10240
9 - 1:20480

* The serogroup reacting at highest titre, presumed to be the infecting one

Table 3. Comparison of results of MAT, PHA and ELISA

Tests	No. Of samples			
	Dog	Cattle	Human	Total
MAT+, PHA+, ELISA+	30	12	56	98
MAT-, PHA-, ELISA-	36	24	52	112
MAT+, PHA-, ELISA-	0	0	0	0
PHA+, MAT-, ELISA-	8	2	2	12
ELISA+, PHA-, MAT-	14	12	12	38
MAT-, PHA+, ELISA+	10	0	4	14
PHA-, MAT+, ELISA+	2	24	24	50
ELISA-, PHA+, MAT+	4	0	4	8
Total samples tested	104	74	154	332

+ indicates positive reaction of the test
- indicates negative reaction of the test

Table 4 Relative sensitivity and specificity of ELISA to MAT and PHA

Tests	Sensitivity	Specificity
MAT	0.9487	0.7045
PHA	0.8485	0.56

Table 5 Relative sensitivity and specificity of MAT to ELISA and PHA

Tests	Sensitivity	Specificity
ELISA	0.74	0.9393
PHA	0.8030	0.75

Table 6 Relative sensitivity and specificity of PHA to ELISA and MAT

Tests	Sensitivity	Specificity
ELISA	0.56	0.8485
MAT	0.6794	0.8523

Table 7. Relative sensitivity and specificity of MAT compared to PHA and ELISA in dog, cattle, pig, rodent and human being

Tests	Sensitivity					Specificity				
	Dog	Cattle	Human	Pig	Rodent	Dog	Cattle	Human	Pig	Rodent
PHA	0.6721	0.7391	0.909	0.625	0.4545	0.95	0.6103	0.7272	0.8529	0.8709
ELISA	0.5714	0.75	0.833			0.9166	1.0	0.9310		

Table 8 Relative sensitivity and specificity of PHA compared to MAT and ELISA in dog, cattle, pig, rodent and human being

Tests	Sensitivity					Specificity				
	Dog	Cattle	Human	Pig	Rodent	Dog	Cattle	Human	Pig	Rodent
ELISA	0.7142	0.25	0.625			0.75	0.9230	0.8965		
MAT	0.9318	0.3617	0.7142	0.5	0.55	0.7402	0.8867	0.9142	0.9062	0.8181

Table 9 Relative sensitivity and specificity of ELISA compared to MAT and PHA in dog, cattle, pig, rodent and human being

Tests	Sensitivity			Specificity		
	Dog	Cattle	Human	Dog	Cattle	Human
MAT	0.888	1.0	0.9523	0.6470	0.6842	0.7714
PHA	0.7692	0.8571	0.909	0.6923	0.4	0.5909

Table 10 MAT titres of positive sera to reference serogroups of *Leptospira*

Reference serogroups of *Leptospira*	Sera samples				
	293	**121**	**576**	**R9**	**TB12**
Icterohaemorrhagiae	1:320	1:5120	1:2560		
Pomona	1:2560	1:20480	1:5120	1:80	
Australis	1:20480	1:160	1:1280		
Patoc	1:2560		1:2560	1:80	
Canicola	1:1280				
Grippotyphosa		1:10240		1:2560	
Sejroe					1:20480
Autumnalis					1:40

Table 11 MAT titres of positive sera to local isolates of *Leptospira*

Isolate No.	Sera Samples used for MAT				
	293	**121**	**576**	**R9**	**TB12**
289	1: 320	1:1280			
BT	1:5120	1: 10240	1:640		
M64	1:640	1:2560	1: 640	1:80	
R13	1:5120		1:320		

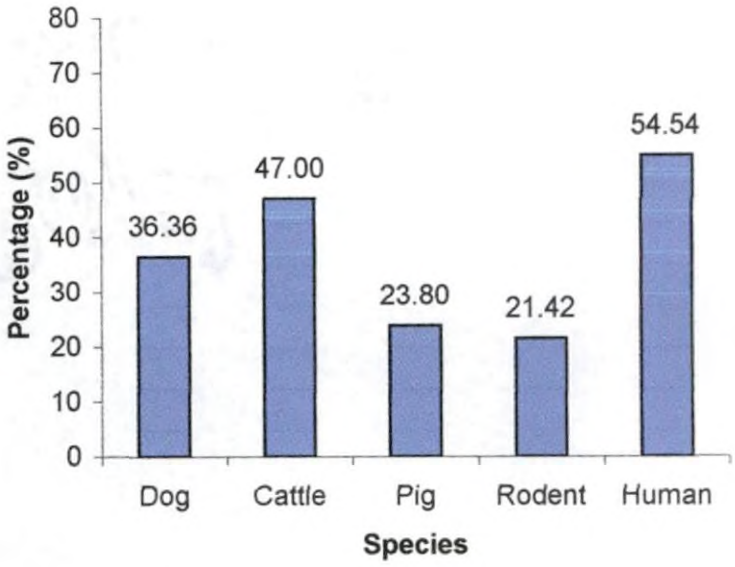


Fig. 1 Species wise seroprevalence of leptospirosis by MAT

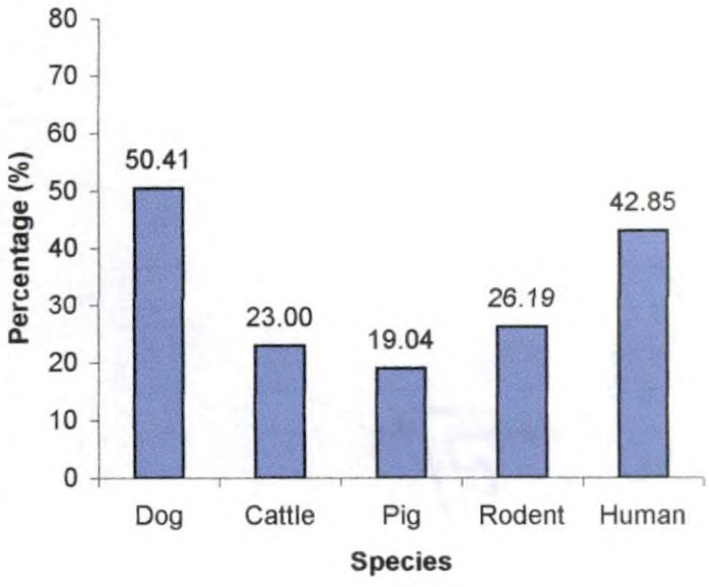


Fig. 2 Species wise seroprevalence of leptospirosis by PHA

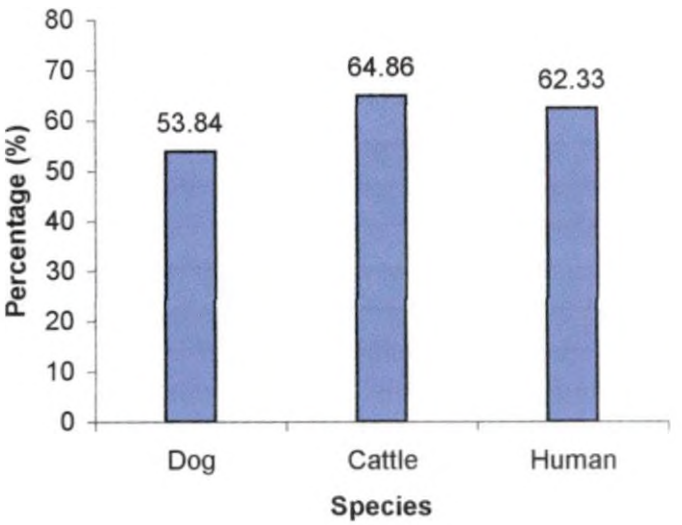
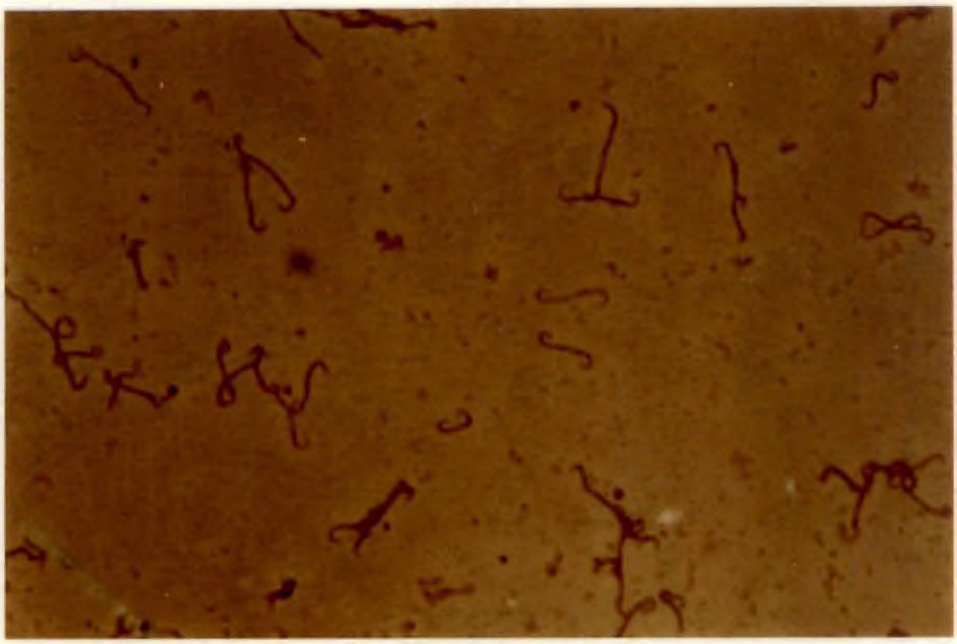
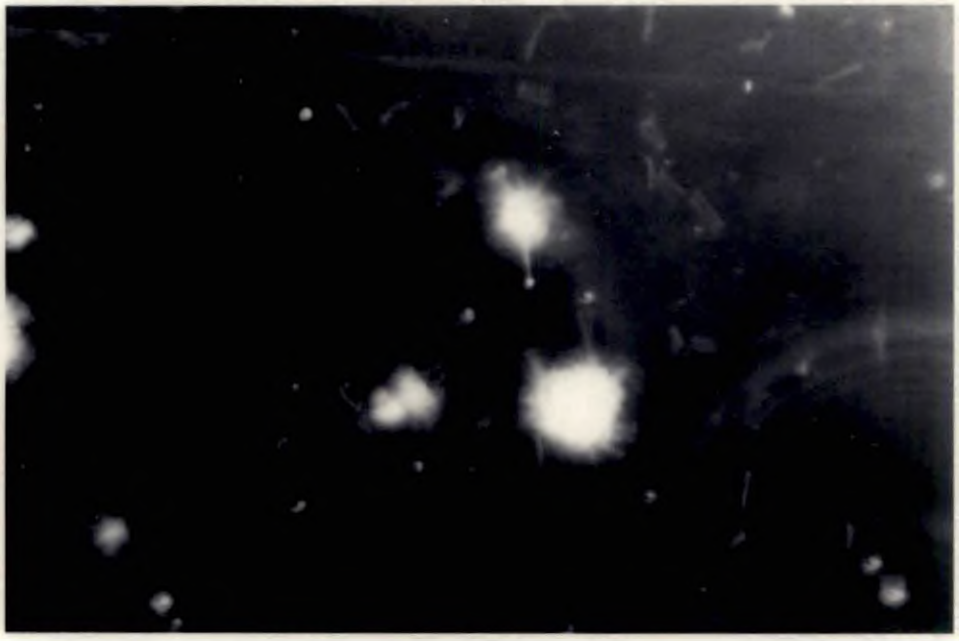


Fig. 3 Species wise seroprevalence of leptospirosis by ELISA



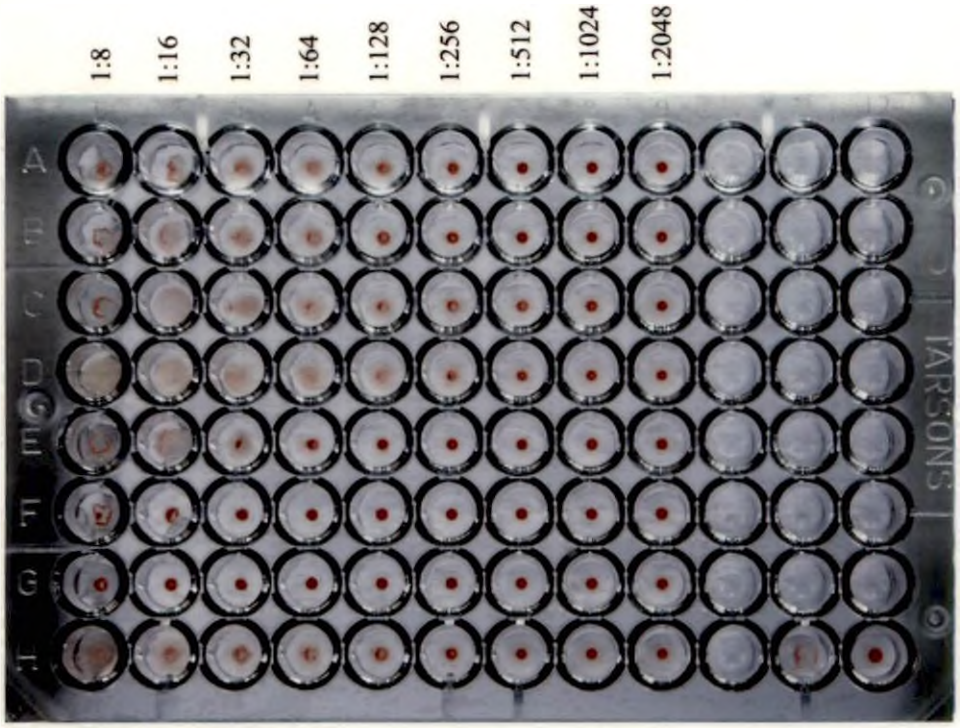
A



B

Plate 1. (A) Fontana's staining of leptospires (1000X)

(B) Agglutination of leptospires in MAT by DFM (450X)



A

B

Plate 2. (A) Passive haemagglutination assay
H 11 Positive control
H 12 RBC control

(B) Indirect ELISA

Wells H 11 & H12 - Conjugate control
G 11 & G12 - Negative control
F 11 & F12 - Positive control
All other wells - Test sera in duplicates

Discussion

DISCUSSION

Leptospirosis, a multisystem zoonotic disease of public health importance and economic significance is caused by infection with pathogenic bacteria of genus *Leptospira*. The leptospires though not particularly host specific, each serovar is believed to have a reservoir or carrier host in which subclinical infection is associated with prolonged leptospiruria. For most leptospires, the carrier hosts are rodents, but some serovars like *pomona, tarassovi* and *hardjo* appear to be adapted to domestic animals (Sullivan, 1974). Human beings are considered as end hosts, who acquire the infection almost exclusively from sources originating in animals (Faine, 1998).

Epidemics of leptospirosis are being increasingly reported from various parts of this country, with most outbreaks occurring during the rainy seasons (Bharadwaj *et al.*, 2002). For adopting a suitable control measure for the disease, an accurate knowledge of the epidemiology of infection in human beings and animals is required.

Diagnosis of leptospirosis based on clinical presentation and epidemiological background alone is difficult, as this disease may mimic other unrelated infections like influenza, meningitis, hepatitis and dengue or viral haemorrhagic fever. Laboratory diagnosis is primarily based on isolation of the pathogen from biological specimens or demonstration of a rise in serum antibody titre (Saengjaruk *et al.*, 2002).

The present study was carried out, with this perspective, to assess the role of animals in the epidemiology of human leptospirosis, by detecting the prevalence of leptospiral infection in animals and man in and around Thrissur, by serology and isolation.

Isolation

Attempts were made to isolate *Leptospira* from canines and human beings during the acute and convalescent stages of infection, so as to establish the presence of infection in the population. Isolation from murines was also tried in order to reveal the carrier status of these animals.

Isolation trials were done mostly in Fletcher's semisolid media supplemented with 10 per cent rabbit sera (FSMR) .The EMJH media could not be used for routine culture due to scarcity of BSA required in the preparation of the EMJH enrichment.

Blood samples collected from 15 canines and 30 human beings, during the acute phase of illness, were tried for isolation. Out of this *Leptospira* could be isolated from one human blood sample in Fletcher's semisolid medium (FSMR).

Venugopal *et al.* (1990) reported that Fletcher's and Korthoff's media were superior to EMJH media for isolation of leptospires from human blood. They opined that absence of sera in EMJH media might be responsible for the failure in isolating *Leptospira* in EMJH media. The inability to isolate *Leptospira* from infected human beings could also be attributed to administration of antibiotics by local practitioners before the patients were referred to the hospitals (Bharadwaj *et al.*, 2002). In the present study, this could have been a possible reason for the reduced number of isolations from human beings.

Leptospires are most frequently isolated from kidney specimens of animals (Anderson *et al.*, 1993). In this study, kidney and liver tissues of an autopsied German Shepherd Dog, cultured in Fletcher's semisolid media (FSMR), showed a subsurface opacity similar to leptospiral growth. The few leptospires that grew in the primary culture, failed to grow in subsequent subcultures. This could have been due to some nutritional inadequacy in the particular batch of media used for culture.

Leptospira could not be isolated from any of the canine blood and urine samples collected from serologically positive dogs. Harkin *et al.* (2003) failed to isolate *Leptospira* from any of the PCR positive canine urine samples subjected to isolation trials.

As the leptospires are fastidious organisms which require rich media at neutral pH for their growth, isolation trials are usually predisposed to problems of contamination (Johnson and Rogers, 1964), especially while culturing leptospires from unsterile sources like urine and aborted fetal tissues (Adler *et al.*, 1986).

Fluoro-uracil, an analogue of uracil is very active in inhibiting growth of many bacteria while supporting growth of leptospires. But once contaminating organisms start growing in the 5-FU medium, addition of higher concentrations of 5-FU has no effect (Johnson and Rogers, 1964). In this study, urine samples were cultured in media containing 5-FU, which effectively controlled the growth of contaminants present in the inocula.

Carlos *et al.* (1971b) could isolate *Leptospira* from canine blood samples in Fletcher's semisolid media with 5-FU while Kaveri and Upadhye (1981) isolated leptospires, from canine urine, collected by catheterization, in EMJH media with 5-FU.The inability to isolate *Leptospira* from any of the canine samples in this study could have been due to low number of viable organisms in the inocula or due to administration of antibiotics prior to isolation trials.

In an attempt to detect the natural reservoir hosts amongst rodent species, 11 bandicoots and 31 rats, were caught from various places in and around Thrissur. Bandicoots and rats are reported to be important reservoir hosts of *Leptospira* among rodent species (Pargaonker, 1957; Adinarayanan and James, 1980).

Sera samples from all these 42 rodents were subjected to serologic testing by MAT and PHA. Isolation was tried from 35 rodents only. Kidney tissues (35) and urine samples (4) collected from these rodents were cultured in Fletcher's

semisolid media (FSMR). Portions of kidney cortex punched out with Pasteur pipettes were inoculated directly into media and serially diluted into three or four tubes. Leptospires could be isolated from the kidney tissues of five rodents .Of these three were from bandicoots and two from rats.

De Lange *et al.* (1987) reported enhanced isolation rates from porcine kidney tissues, following serial dilution of tissue specimens. They opined that this was probably due to the dilution effects on the toxins, released from the tissues by autolysis, and the contaminant organisms that compete with the leptospires. However in this study, it was found that primary isolation of *Leptospira* was possible mostly in those culture tubes in which the tissue pieces were present.

The first report of isolation of *Leptospira* from murine species in India was made by Adinarayanan and James (1980) from Thrissur. They could isolate *Leptospira* from eight out of 46 bandicoots and one out of the 114 rats examined, in Fletcher's semisolid media with 10 per cent rabbit sera. Natarajaseenivasan and Ratnam (1997) reported the isolation of *Leptospira* from one out of 34 rodents subjected to isolation trial in EMJH media.. Gangadhar and Rajasekhar (1998) could isolate *Leptospira* in EMJH semisolid media from 89 of the 500 rodents subjected to isolation trials.

Serology

A number of serological techniques, each having its own sensitivity and specificity have been developed for the diagnosis of leptospirosis. For a reliable diagnosis, it is necessary to use more than one test together or successively.

The commonly used laboratory techniques are MAT and ELISA of which MAT is the standard reference test. The PHA is useful as a screening test for leptospirosis in the early stages of infection (Levett and Whittington, 1998). It is considered as a relatively easy and sensitive test.

MAT

The MAT was done on sera samples from 121 dogs, 100 cattle, 84 pigs, 42 rodents and 154 human beings.

Sera samples were initially screened at 1:80 dilution against all reference serovars, and those sera with agglutinating activity at 1:80 dilution were retested by making serial two fold dilutions to a maximum dilution to 1:20480. The prevalence rates detected by MAT were 36.36 per cent in canines, 47 per cent in bovines, 23.8 per cent in porcines, 21.42 per cent in murines (rats and bandicoots) and 54.54 per cent in human beings.

Serogroup *Autumnalis* could be used in the screening of 43 bovine sera samples only.Though *L.autumnalis* is reported to be one of the common serogroups prevalent in Kerala (Kuriakose *et al.*, 1997) and Tamil Nadu (Natarajaseenivasan *et al.,* 2002), in this study, only four of the 43 samples tested were positive to this serogroup .

Dog

Agglutinins to *L. pomona* and *L. australis* were detected in 38.63 per cent and 36.36 per cent of positive cases respectively. Agglutinins to *L. canicola* (6.8 per cent), *L. icterohaemorrhagiae* (6.8 per cent), *L. patoc* (4.5 per cent) and *L. grippotyphosa* (2.3 per cent) were detected in few cases only. Maximum titre of 1:5120 was detected against *L. Pomona* and *L. australis*.

Canine leptospirosis is widely prevalent in various parts of the country, as it is evident by serological survey (Verma, 1982), and infections in dogs caused by *L. icterohaemorrhagiae* and *L. canicola* are recognized through out the world (Sullivan, 1974).

The present study detected *L. pomona* and *L. australis* as the most prevalent serogroups infecting dogs in Thrissur. In an earlier serological study conducted on canine leptospirosis, by Indu (1997), *L. pomona* was found to be

the most prevalent serogroup in in dogs in Thrissur. Leptospiral agglutinins were detected in 32.62 per cent of the dogs examined. Prevalence of agglutinins to *L. pomona* in dogs was also reported by Morter *et al.* (1959). They opined that high rate of *L. pomona* infection among dogs could be correlated to their close contact with livestock.

In the present study, infections due to *L. canicola* and *L. icterohaemorrhagiae* were detected in very few cases only. This could have been due to the routine vaccination of dogs against these serovars.

Cattle

The present study revealed a prevalence rate of 47 per cent in cattle by MAT.

Leptospira hardjo of serogroup *Sejroe* was found to be the most prevalent serovar in this study, with 42.55 per cent of the positive cases reacting to this serovar. *Leptospira pomona* and *L. patoc* were detected in 12.76 per cent and 19.14 per cent of the positive cases respectively. Maximum agglutination titre of 1:20480 was detected against serogroup *Sejroe*.

The results of this study substantiated the previous serologic studies (Batra *et al.*, 1990; Ramakrishna and Venkataraman, 1994; Ciceroni *et al.*, 1995), which indicated that serovar *hardjo* of serogroup *Sejroe* was the most prevalent serovar affecting cattle.

Natarajaseenivasan and Ratnam (1997) reported a prevalence rate of 61.5 per cent in cattle in a village in Tamil Nadu, with equal distribution to serogroups *Sejroe*, *Pomona* and *Autumnalis* (20 per cent). Mrunalini and Ramasastry (2000) detected subclinical leptospirosis in apparently healthy cattle caused by *L. hardjo* and *L. andamana*.

Pig

MAT detected a prevalence rate of 23.80 per cent in pigs with 40 per cent of the positive cases having agglutinins to serogroup *Pomona. Serogroup Grippotyphosa* was detected in 30 per cent of the positive cases while serogroups *Canicola* and *Icterohaemorrhagiae* were detected in 20 per cent and 10 per cent of positive cases respectively.

In India porcine leptospirosis caused by serovar *pomona* has been reported by several authors (Bhagwat, 1964; Rajasekhar and Nanjiah, 1971). *Leptospira pomona* is reportedly the most important serovar affecting pigs and is responsible for abortions and stillbirths in susceptible gilts and sows (Chappel *et al.,* 1992). *Leptospira grippotyphosa* infection in sows, producing abnormal litters, was detected by Hanson *et al.* (1971).

Of the 20 positive sera samples, 16 were found to have titres lower than 1:160. The low MAT titres (1:256 or lower) could have been due to the fact that the pigs were in the early phase of infection or might be associated with a past infection (Chappel *et al.*, 1992). The highest agglutination titre of 1:640 was detected against serogroup *pomona.*

Pigs have a special significance in the epidemiology of leptospirosis because of the high intensity and long duration of leptospiruria (Sullivan, 1974). Pig and cattle are considered to be ideal hosts for the perpetuation of *L. pomona.* Hence these hosts could be an important source of *L. pomona* infection to dogs (Morse, 1960).

Rodents

The MAT as a serodiagnostic technique in rodents has many disadvantages. The amount of serum required for MAT cannot always be obtained from small animals like rodents. It is also difficult to decide the cut off titre for MAT in rodents. Different titres have been considered to clarify a result

as positive, ranging from 1:10 to 1:100 with intermediate criteria of 1:20, 1:40 and 1:50 (Vanasco *et al.*, 2001).

In the present study the cut off titre for MAT was taken as 1:20 as per the findings of Vanasco *et al.* (2001) and MAT detected antibodies, at significant titres, in 21.42 per cent of the rodents examined.

Leptospira pomona was found to be the most prevalent serovar (44.4 per cent), followed by *L.icterohaemorrhagiae* and *L.australis* (22.2 per cent). The agglutination titres ranged from 1:20 to 1:20480. The highest titre of 1:20480 was recorded against serogroup *Pomona* while lowest titre of 1:20 was recorded against serogroups *Australis* and *Canicola.* The prevalence of *L. pomona* infection in rodents is particularly relevant as this is an emerging serovar in many parts of the world.

Leptospira pomona infection in rodents in Tamil Nadu was reported by Natarajaseenivasan and Ratnam (1997). They recorded a prevalence rate of 52.9 per cent, with the highest titre of 1:160 against *L. pomona* and *L. autumnalis.* Natarajaseenivasan *et al.* (2002) detected a prevalence of 52.1 per cent in rodents at Salem. The predominant serogroup was reported to be *Autumnalis* (26.6 per cent), with a maximum titre of 1:160, followed by serogroup *Icterohaemorrhagiae* (21.7 per cent). The cut off titre for MAT was taken as 1:20.

Human beings

Out of the 154 samples tested, 84 were positive for antibodies to *Leptospira* (54.54 per cent). The most prevalent serogroup was *Australis* (42.85 per cent), followed by serogroup *Pomona* (28.57 per cent). Highest agglutination titre of 1:20480 was recorded against *L. pomona* and *L.australis.* Antibodies against serogroups *Patoc, Pyrogenes, Icterohaemorrhagiae, Grippotyphosa* and *Canicola* were also detected.

Leptospira australis was identified as the most prevalent serovar in human beings by Murhekar *et al.* (1998) who detected an overall prevalence rate of 54.2 per cent for human leptospirosis in Diglipur District of North Andaman. Rathinamsivakumar *et al.* (1996) demonstrated leptospiral infection in patients with recurrent ophthalmic involvement in Tamil Nadu, caused predominantly by serovar *pomona* and also by serovars *australis, autumnalis* and *javanica.*

In this study, MAT revealed the presence of a common serogroup infecting man, animals and rodents, namely *L. pomona.* Rodents could be considered as a source of serovar *pomona* infections in grazing animals, which in turn contributed to infection in human beings (Natarajaseenivasan and Ratnam, 1997).

Serogroup *Australis* was also found to be a common infecting serovar, especially in human beings and canines, but none of the porcine sera were found to be positive to this serogroup. Hence it could be possible that most of the human infections caused by *L. australis* occurred through close contact with dogs.

The MAT should be ideally performed on paired sera samples and the criterion for a definitive diagnosis of current leptospirosis is a four-fold rise in titre or sero conversion (Vijayachari *et al.*, 2001b). Bharadwaj (2002) detected 15.9 per cent positivity by MAT when acute phase sera samples alone were examined, while 43.78 per cent positivity was obtained when acute as well as convalescent phase sera were examined. Faine (1998) reported that in a non-endemic area, the level of antibody, however low, might signify leptospirosis in the first week of illness. But in an endemic area, a minimum titre of 1:400-1:800 is required for the infection to be considered significant.

In this study, the cut off titre for MAT was taken as 1:80 in canine, bovine, porcine and human sera. As paired sera samples could not be examined in this study it is difficult to point out whether the antibodies detected in sera

samples, having titres ranging from 1:80 to 1:400, were due to present illness or past one.

Microscopic Agglutination Test which is considered as the standard reference test for leptospirosis, has several disadvantages. The requirement for paired sera samples for an accurate result, delays diagnosis, while false negative results, that occur when the causative serogroups are not included in the panel of reference leptospires, leads to incorrect diagnosis.

Passive Haemagglutination Assay

Prevalence of leptospirosis in dogs, cattle, pigs, rodents and human beings as detected by PHA were 50.41per cent, 23 per cent, 19.04 per cent, 26.19 per cent and 42.85 per cent respectively.

A maximum titre of 1:512 was recorded against canine and human sera while in cattle a maximum titre of 1:128 was recorded. In rodents and pigs, the maximum titre recorded was 1:32. Srivastava *et al.* (1985) detected a maximum PHA titre of 1:64 in cattle, using sonicated leptospiral antigen. They opined that titres of 1:16 or below indicated an infection, which might have occurred two months earlier whereas titres of 1:32 and 1:64 indicated recent infection within two months.

Fresh unmodified sheep erythrocytes were used in preparation of the antigen in this study. Gluteraldehydre fixed tanned erythrocytes were not used in this study as it gave aberrant test results.

Palit *et al.* (1974) suggested that, in PHA, the substance used for sensitization of unmodified red cells consisted mainly of carbohydrate (CHO) antigens while those, which could sensitize tanned red cells involved protein antigens. They found that the ethanol precipitated antigen (ESS) contained large amount of CHO (17.18 per cent w/w) and much less of protein (4 per cent w/w).

They also pointed out that the CHO moiety was largely responsible for the genus specificity of the test.

Hence in the present study, the use of fresh unmodified SRBCs in PHA, helped in effective screening of leptospirosis in man and animals, because of the genus specific nature of the antigen used for sensitizing the SRBCs.

The PHA was carried out using ethanol extracted antigen from serovars *pyrogenes* and *patoc*. Morris *et al.* (1977) examined the antibodies active in IHA, using ethanol extracted antigen, in bovine antisera and found that most of the antibodies active in IHA were in the IgM fraction. Terpstra *et al.* (1985) opined that the IgG antibodies even if they bind to ESS might agglutinate the sensitized erythrocytes poorly in comparison with IgM, which had more active binding sites. Hence it could be assumed that sera samples that showed high PHA titres in this study were from acute cases of leptospirosis.

The optimum dilution of the antigen used in this study was 1:2. The antigen was prepared from leptospires grown in 100 ml volumes of EMJH media. This was in agreement with the results obtained by Dwarki (1978) who used a 1:3 dilution of the antigen prepared from two litres of EMJH liquid media and Nair (1980) who used a 1:4 dilution of the ESS prepared from litre volumes of Korthoff's media.

Nair (1980) reported that the ethanol extracted ESS could be stored at – 20°C for atleast three months without any change in its sensitivity. In the present study, it was found that ESS could be stored only for a maximum of five days at 4°C following which aberrant test results were obtained. As the sensitized SRBC antigen used in this study was prepared from fresh and unfixed SRBCs, it could only be stored for a maximum period of five days at 4°C.

ELISA

Indirect ELISA carried out in this study detected a prevalence of 53.84 per cent in dogs, 64.86 per cent in cattle and 62.33 per cent in human beings.

The ELISA was carried out as per Terpstra *et al.* (1985), with minor modifications. The antigen used was the heat extracted antigen as per protocol, while the chromogen used was OPD. The OD values were read at 492 nm in an ELISA reader. An IgG ELISA for diagnosis of human leptospirosis was developed by Natarajaseenivasan *et al.* (2002), using the heat extracted antigen and OPD as chromogen. This ELISA could detect leptospiral antibodies in 71.1 per cent of mill workers at Salem district.

Terpstra *et al.* (1985) reported that a major advantage of the heat extracted antigen was its stability. They found that it could be stored for long periods in liquid state and also coated on to polystyrene plates. The heat extracted antigen used in this study could be stored for two to three weeks at 4°C without any deterioration in its antigenicity, but when coated on to polystyrene microtitre plates, it could be stored only for a maximum period of one week at room temperature, following which it gave aberrant test results. This could be due to excessive drying of the plates caused by the high ambient temperature.

An indirect ELISA for detection of leptospiral antibodies in dog sera was developed by Ribotta *et al.* (2000) using the heat extracted antigen. Lottersberger *et al.* (2002) developed an IgG ELISA using sonicated leptospiral antigen, from cultures of serovar *hardjo,* for detecting leptospiral antibodies in bovine sera.

Comparison of serologic tests

The results of the three tests were analysed by use of kappa (κ) statistics. The percentage of agreement between MAT, PHA and ELISA showed kappa values above 0.9 for all the species tested. As per Raj *et al.* (1998), κ values above 0.81 indicated perfect agreement whereas κ values below 0.1 indicated poor agreement.

Of the 332 sera samples subjected to the three tests, 98 were tested positive by all the three tests while 112 tested negative by all the tests.

The ELISA could detect leptospiral antibodies in 38 sera samples that were negative by both MAT and PHA, while only eight of the ELISA negative sera samples gave positive results by MAT and PHA.

The heat extracted antigen used in this study is reported to contain four antigenic fractions of different specificities, while the ethanol extracted antigen used in PHA contained only a single antigenic fraction. This antigen is also supposed to contain serogroup or type specific fractions, apart from the broadly reactive genus specific fractions (Terpstra *et al.*, 1985). Ribotta *et al.* (2000) opined that MAT negative, ELISA positive sera samples were obtained due to the presence of non agglutinating leptospiral antibodies which were detectable by ELISA, and not by MAT, which could detect only agglutinating antibodies. Thiermann and Garrett (1983) reported that somatic antigenic components played a role in ELISA while MAT reaction was mainly based on agglutination of surface antigenic components present on live organisms. All the above mentioned factors could have contributed to the better sensitivity of ELISA compared to MAT and PHA in this study.

The PHA could detect antibodies in 12 sera samples, which were negative by MAT and ELISA. Several authors have identified IgM as the major class of immunoglobulin detected by PHA (Palit and Gulasekharam, 1973; Morris *et al.*, 1977). In addition to this, the ability of PHA to detect both IgM and IgG antibodies was reported by Levett and Whittington (1998). The ELISA could detect IgG antibodies only, as the conjugate used contained anti IgG antibody. Considering the fact that ELISA is a highly sensitive test, it could be possible that those sera samples, which were negative by ELISA and positive by PHA, contained more of IgM antibodies and hence were acute phase sera.

Srivastava *et al.* (1985) reported that human beings became positive for IHA much earlier than for MAT and that no such phenomena was observed in animals. However in this study out of the 12 PHA positive sera samples which

were negative by MAT, eight were of dogs while only two were of human beings.

In this study, MAT did not detect leptospiral antibodies in any of the sera samples, which were negative by ELISA and PHA. This showed the high specificity of MAT compared to PHA and ELISA. Microscopic agglutination test is recognized as the 'Gold standard technique' for serodiagnosis of leptospirosis and false positive results are very rare in case of MAT.

Eight samples, which were negative by ELISA, gave positive results by MAT and PHA. This could be because ELISA detected only IgG antibodies while PHA detected mostly IgM antibodies and MAT could detect both IgG and IgM antibodies.

Fourteen of the MAT negative sera samples were tested as positive by ELISA and PHA. The MAT is a serogroup specific test. If the infecting *Leptospira* serogroup is not included in the battery of reference leptospires used in the test, it could lead to false negative results. In this study only 10 reference serogroups were used for conducting MAT. Hence it is possible that some of the infecting serovars were not included in this study, which led to false negative results. Nevertheless, the possibility that sera gave false positive results by ELISA and PHA due to presence of cross reacting antibodies cannot be ruled out (Ribotta *et al.*, 2000). The antibodies detected by PHA apparently contributed little to the agglutination titres of whole *Leptospira* in the sera and there is reportedly little correlation between MAT and PHA titres, except during the initial phase of infection (Morris *et al.*, 1977). Hence it is not necessary that sera samples that gave positive results by PHA be positive by MAT.

Fifty samples that were tested as negative by PHA gave positive results by ELISA and MAT. This could be because these sera samples contained mainly IgG antibodies and hence were poorly detected by PHA. Morris *et al.* (1977) reported that PHA titres of bovine antileptospiral sera were low and specific IgM,

the major class of antibody detected by the test, was unlikely to persist in infected cattle. This could be the reason why in this study, 24 of the total 74 bovine sera samples, which gave negative results by PHA tested positive by MAT and ELISA. This could also be the reason why none of the MAT negative bovine sera samples gave positive results by ELISA and PHA and none of the ELISA negative bovine sera samples gave positive results by PHA and MAT.

Sensitivity and specificity

Considering MAT as the gold standard test, the relative sensitivity and specificity of ELISA and PHA to MAT were analyzed by kappa statistics.

The overall sensitivity and specificity of IgG ELISA was calculated as 0.9487 and 0.7045 respectively.

The relatively specificities of IgG ELISA were similar in dog, cattle and human beings, ranging from 0.64 to 0.77. Ribotta *et al.* (2000) reported 100 per cent sensitivity and 95.6 per cent specificity for IgG ELISA relative to MAT in dogs. Natarajaseenivasan *et al.* (2002) reported greater sensitivity of IgG ELISA to MAT in detecting leptospiral antibodies in human sera.

It was seen that the relative sensitivity of IgG ELISA to detect leptospiral antibodies in cattle was 1.0 while those in human beings and dogs were 0.95 and 0.88 respectively. The high sensitivity of IgG ELISA in cattle shows the presence of *Leptospira* specific IgG antibodies in sera, which may indicate chronic infection or carrier status of the animal.

Relative sensitivity of PHA to MAT was found to be maximum in dogs (0.9318) and least in cattle (0.3617) in this study. This may indicate the stage of infection in these animals. Dogs are usually prone to acute infection while cattle are reportedly chronic carriers of leptospiral infection

The PHA showed maximum specificity in detecting leptospiral antibodies in human beings and pigs (approximately 0.9) followed by cattle and rodents

(approx. 0.8) while it showed least specificity in dogs (0.7402). The large number of PHA positive samples detected in dogs may indicate false positive results due to non specific agglutination of sensitized SRBCs in dog sera.

Levett and Whittington (1998) reported 100 per cent sensitivity and 94 per cent specificity for IHA to MAT in human sera while Sulzer *et al.* (1975) revealed a relative sensitivity and specificity of 92 per cent and 95 per cent respectively for IHA in human sera.

Serotyping of isolates

Serogroup identification of isolates is usually done by MAT, using rabbit antisera (group sera) raised against all known pathogenic serogroups (Venugopal *et al.*, 1990; Sehgal *et al.*, 2000).

In the present study due to lack of availability of positive sera against the known *Leptospira* serogroups, field sera, which had presented high MAT titres to individual reference serogroups, were used to define tentatively the serogroups of the local isolates.

Field sera nos. 121, 576 and 293 were found to have high titres to the reference serogroups *Pomona* and *Australis*. Serum no. 293 had maximum titre to serogroup *Australis* while sera nos. 121 and 576 had maximum titres to serogroup *Pomona*. Sera nos. R9 and B12 showed maximum agglutination titre to serogroups *Grippotyphosa* and *Sejroe* respectively.

On carrying out MAT using the local isolates as antigen, it was found that isolates M64 and BT showed agglutination at high dilutions in sera nos .121 followed by 293 and 576 whereas isolate R13 showed high titres in serum no. 293 followed by serum no.576. The isolates M64, BT and R13 failed to agglutinate in serum no. TB12.

These findings suggest that the three isolates BT, M64 and R13 probably belonged to the serogroups *Pomona* or *Australis*. Isolates BT and M64 showed

maximum agglutination titres in serum no. 121 which had tested positive for serogroup *Pomona* whereas isolate R13 showed maximum agglutination in serum no. 293 which was positive for serogroup *Australis*. From this we could conclude that isolates BT and M64 from bandicoots probably belonged to serogroup *Pomona* while isolate no. R13 from rat probably belonged to serogroup *Australis*.

Isolation of serogroups *Pomona* and *Icterohaemorrhagiae* from rats has been reported by Thiermann (1977). Several authors have also reported isolation of serogroup *Javanica* from rodents (Natarajaseenivasan and Ratnam (1997); Gangadhar and Rajasekhar, 1998).

The isolate no. 289 showed low agglutination titres in serum no. 121 (1:1280), which was positive for *L. Pomona and* serum no. 293 (1:320), which was positive for *L. australis,* but did not agglutinate in serum no. 576. Hence no inference on its serogroup identity could be made. Kuriakose *et al.* (1997) in a study conducted at Kolenchery, Kerala could isolate a new serovar belonging to serogroup *Australis*, from a human patient, and named it as serovar *australis bharathy*.

Serovar identification of local isolates of *Leptospira* following serogroup identification is usually done by cross agglutination absorption test using antisera raised against serovars of identified serogroups (Sehgal *et al.*, 2000).

Comparison of serological tests to culture

The serum sample obtained from the human patient who was culture positive (Isolate No. 289) tested negative by MAT, PHA and ELISA. Absence of antibodies to *Leptospira* in the serum of the patient, from whom isolation was made, is paradoxical. However isolation of leptospires from cattle and human beings without detectable antibody titres has been reported earlier (Anderson *et al.*, 1993). One of the reasons for this could be that protective antibodies were not produced in the patient's body at the time of collection of serum .In this case, the

serum for serologic testing was separated from the same blood, which was subjected to isolation trials.

The bandicoot and two rats from which M64, M63 and R13 were isolated, tested serologically negative by all the three tests. Serum of the rat from which M63 was isolated, showed a PHA titre of 1:8, which was also considered as negative. This phenomenon of isolation positive, serologically negative rodents have been observed repeatedly by many authors (Shotts *et al.*, 1975; Thiermann, 1977).

The rodents are susceptible to acute infection only during the first few days after birth. In about 10 days time, the immune system develops and they become resistant to further infection (Faine, 1998) and the leptospires get lodged in the renal tubules of the rodent for their life time. This could be the reason for the presence of low amount of antibody in the sera of these rodents, though leptospires were isolated from their kidneys.

The bandicoot from which BT was isolated was found to be seropositive by MAT, with high titres of 1:20480 to serogroup *Pomona* and 1:10240 to serogroup *Patoc*. But it gave negative results by PHA. The serum of the bandicoot from which R6 was isolated, showed a titre of 1:32 by PHA and a titre of 1:80 to serogroup *Pomona* by MAT. Vanasco *et al.* (2001) could detect culture positive rodents as serologically positive by ELISA and also by MAT at a titre of 1:50 and above.

The present study could successfully detect antibodies to *Leptospira* in the sera of human beings, the end hosts, animals, the propagative hosts and rodents, the reservoir hosts of *Leptospira*. The evidence of serology was substantiated by successful isolation of *Leptospira* from the end hosts as well as the reservoir hosts.

The three serological tests could diagnose the disease with varying degrees of sensitivity and specificity. Isolation of *Leptospira* from rodents,

revealed the reservoir status of these animals, while isolation from the human patient confirmed the presence of infection in the population. It is possible that the rodents living in the premises of cattle sheds and households acted as a source of infection to animals, which in turn contributed to infection in human beings, with whom they had close contact.

The MAT proved to be the most appropriate test for epidemiological surveillance as it could be applied to any animal species and gave a general impression about the serogroups prevalent in the population. In this study, the MAT detected *L. pomona* and *L. australis* as the common serovars infecting human beings, animals and rodents.

The IgG ELISA was found to be of particular value in rapid screening of the population for leptospirosis and in the diagnosis of chronic infections while the PHA was found to be a valuable assay for diagnosis of acute leptopirosis. Compared to these tests, MAT was found to be highly cumbersome and time consuming.

Based on the limited data obtained from this study, a future, more elaborate study on the epidemiology of leptospirosis in Kerala could be carried out, to identify the important serovars of *Leptospira* which are responsible for the endemicity of the disease in the state and to suggest a foolproof control measure for leptospirosis.

Summary

SUMMARY

Leptospirosis refers to a number of disease syndromes in animals and man caused by infection with pathogenic leptospires. The disease is reported to have world wide distribution and is considered as a major public health problem especially in the tropics. Many places in India are known to be endemic for leptospirosis, while in Kerala, several districts witness yearly spurts in cases of human and animal leptospirosis with heavy casualty, during the post monsoon periods. The control measures undertaken against this disease is so far not successful due to the paucity of a sound knowledge of the correct epidemiology of the infection in this region.

The current study was taken up to assess the role of domestic and peridomestic animals in the causation and propagation of leptospiral infection in animal and human population.

Sera collected from dogs, cattle, pigs, rodents and human beings were screened for the presence of specific antibodies against *Leptospira* by serological tests like MAT, PHA and Indirect IgG ELISA.

For detection of current infection, clinical samples from suspected cases of leptospirosis were tried for isolation of leptospires in Fletcher's/EMJH semisolid media with enrichment.

To determine the role of rodents, specifically rats and bandicoots, in the area, in the maintenance and shedding of leptospires, rats and bandicoots were captured from the premises of animal sheds and households and examined for *Leptospira* by culture and/or serology.

A total of 501 sera samples, collected from normal as well as infected dogs, cattle, pigs, rodents and human beings, were subjected to serologic testing by MAT, PHA and ELISA.

The MAT detected a prevalence of 36.36 per cent in canines, 47 per cent in bovines, 23.8 per cent in porcines, 21.42 per cent in murines and 54.54 per cent in human beings.

The maximum agglutination titre of 1:20480 was detected in cattle and rodents against serogroups *Sejroe* and *Pomona* respectively, and in human sera samples against serogroups *Australis* and *Pomona*. Titres of 1:5120 and 1:640 were detected in canine sera against serogroups *Pomona* and *Australis* and in porcine sera against serogroup *Pomona* respectively. The cut off titre for MAT was taken as 1:80 in all species except rodents. A titre of 1:20 was considered as the cut off in rodents.

The prevalence rate as detected by PHA was 50.41 per cent in dogs, 23 per cent in cattle, 19.04 per cent in pigs, 26.19 per cent in rodents and 42.85 per cent in human beings. The maximum agglutination titre of 1:512, for PHA, was recorded in canines and human beings, while in cattle, the highest titre recorded was 1:128.A titre of 1:32 was the maximum titre detected in porcine and rodent sera. Except in rodents, the cut off titre for PHA was taken as 1:32. In rodents, the cut off was fixed at 1:16.

The ELISA could detect leptospiral antibodies in 53.84 per cent of dogs, 64.86 per cent of cattle and 62.33 per cent of human beings examined.

Of the three tests, ELISA was found to be the most sensitive, but lacked the serogroup specificity of MAT. The MAT proved to be a highly specific test as none of the sera samples which had tested negative by PHA and ELISA, tested positive by MAT.

The antigen used in I-ELISA was the heat extracted antigen which contained different antigenic fractions with varying specificities. The ethanol precipitated antigen used in PHA contained only a single antigenic fraction, which could detect mostly the IgM class of antibody. Hence PHA was found to be more effective in diagnosis of acute leptospirosis.

Leptospira pomona was found to be the predominant serogroup infecting dogs, pigs and rodents. *Leptospira australis* was identified as the most prevalent serogroup in human beings while *L. sejroe* was the predominant serogroup detected in cattle.

The MAT identified *L. pomona* and *L. australis* as the common serogroups prevalent in canine and human population. It was assumed that human infections occurred mainly through close interaction with carrier animals like cattle, dog and pig and that these animals acquired the infection from reservoir hosts like rodents.

Attempts were made to isolate *Leptospira* from clinical cases of human and canine leptospirosis and from rodents.

Out of the 35 rodents subjected to isolation trials, leptospires could be isolated from three bandicoots and two rats.

Of the 30 human and 15 canine blood samples tried for isolation, leptospires were isolated from one human patient showing symptoms of acute leptospirosis.

The positive results obtained by serology and isolation, in this study, confirmed the prevalence of leptospiral infection in the population.

It was found that MAT was the most appropriate test for epidemiological surveillance as it could identify the infecting leptospires upto the serogroup level. The IgG ELISA proved to be the most suitable test for rapid screening of the population for leptospirosis while PHA was an effective assay for diagnosis of acute infection.

Results of this study indicate a high percentage of positivity in animals which might have contributed for the increased incidence of human leptospirosis in the area. The isolation of leptospires from rats and bandicoots from the area, points at their carrier status and role in the maintenance of leptospires.

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PREVALENCE OF LEPTOSPIROSIS IN ANIMALS IN AND AROUND THRISSUR

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ABSTRACT

A study was undertaken to assess the prevalence of leptospirosis in animals and man, in and around Thrissur.

A total of 501 sera samples, collected from dogs, cattle, pigs, rodents (bandicoots and rats) and human beings were subjected to serologic testing, for detection of *Leptospira* specific antibodies, by MAT, PHA and indirect IgG ELISA. Isolation of *Leptospira* was tried from blood of clinically suspected cases of human and canine leptospirosis and from kidney tissues of rodents.

The study revealed the presence of antibodies against *Leptospira* in human beings and all the four species of animals examined by the three tests employed. The prevalence rates detected by MAT in canines, bovines, porcines, murines and human beings were 36.36 per cent, 47 per cent, 23.8 per cent, 21.42 per cent and 54.54 per cent respectively.

Out of 30 human blood samples subjected to isolation trials in Fletcher's/EMJH semisolid media with enrichment, *Leptospira* could be isolated from a single human patient, in Fletcher's semisolid medium with 10 per cent rabbit serum. Of the 35 rodent kidney tissues tried for isolation in Fletcher's semi solid media, leptospires were isolated from three bandicoots and two rats.

Indirect ELISA was found to be most sensitive, of the three tests,for rapid screening of the population for leptospirosis, while PHA was found to be a fairly good test for diagnosis of acute leptospirosis. The MAT proved to be the most specific test which could also identify the serogroup identity of the infecting *Leptospira. Leptospira pomona* and *L. australis* were detected by MAT as the common serogroups prevalent in man, animals and rodents in this area.

The prevalence of common leptospiral serovars in animals and man, indicated that human beings which were the end hosts for *Leptospira* could have acquired the infection mostly from animals like dogs, cattle and pigs, while the isolation of leptospires from rodents revealed the carrier status of these animals.