SCREENING OF DOGS FOR LEPTOSPIRA USING SANDWICH DOT ELISA

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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 **"SCREENING OF DOGS FOR LEPTOSPIRA USING SANDWICH DOT ELISA"** 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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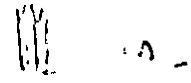
CERTIFICATE

Certified that the thesis entitled **"SCREENING OF DOGS FOR LEPTOSPIRA USING SANDWICH DOT ELISA"** is a record of research work done independently by **Rahul.S.Nair.,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.



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Rahul.S.Nair

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Introduction

1. INTRODUCTION

Leptospirosis is a contemporary zoonosis affecting all warm and even cold blooded vertebrates characterized by fever, severe jaundice and multi-organ failure resulting in severe mortality in untreated cases.

The men at risk to this disease condition includes agricultural workers, animal handlers, veterinarians, abattoir workers, research workers, milkers, housewives, sewage workers and is well related to the recreational and ritual habits of the people. It is an economically important disease of livestock industry due to mortality, reduction in milk yield, stillbirth, abortion and repeat breeding.

The causative agent leptospires are aerobic or microaerophilic gram-negative bacteria slender, helical, motile and members of the order *spirochaetales*. There are more than 230 distinct serovars recognized and these are arranged in 23 serogroups.

The organism exists in the nature through its natural maintenance hosts, the wild and domestic animals. Most of such animals excrete these organisms to their surroundings and will contaminate the various ecological bodies like the water sources, soil etc and will act as a source for the animals and the human beings who accidentally comes in contact with any of these.

Leptospirosis has been described as a zoonosis of protean manifestations. The spectrum of symptoms is extremely broad. The great majority of infections caused by leptospires are either sub clinical or of very mild severity, and patients will probably not seek medical attention.

In human beings the disease can be broadly divided to anicteric type characterized by a febrile illness of sudden onset, headache, myalgia, abdominal pain, conjunctival suffusion, and less often a skin rash and an icteric type which is a much more severe disease in which the clinical course is often very rapidly progressive and this contributes to the high mortality rate, which ranges between five and 15 per cent.

Clinically apparent leptospirosis will be noticed only in animals of economic importance or pet animals where they are close to humans in and around homes or

farms. The severity of the disease ranges from mild sub clinical infection to the much severe forms resulting in loss of production, multi-organ failure and death.

With respect to animals, the infection gains much importance when we need to consider their role as maintenance host of leptospires in nature. The disease is maintained in nature by chronic infection of the renal tubules of these maintenance hosts. The most important maintenance hosts are small mammals, which may transfer infection to domestic farm animals, dogs, and humans.

Leptospirosis is now regarded as a potential public health problem with a much greater incidence in tropical regions and has now been identified as one of the emerging infectious diseases. The epidemiology of leptospirosis has been modified by changes in animal husbandry, climate, and human behavior. Resurgent interest in leptospirosis has resulted from large outbreaks that have received significant publicity.

In the Indian scenario leptospirosis has been recognized as an important emerging disease in the 1980s and 1990s with increased occurrence in Andamans, Tamilnadu and Kerala (Angnani, 2003). Higher incidence of the disease is always seen associated with the monsoon or post monsoon period which claims a sizable number of both human and animal lives. The role of animal maintenance hosts in this scenario is of paramount importance.

Dogs are said to be the renal carriers of leptospires and the contamination of the surroundings with this infected urine can serve as a potential public health hazard. So the detection of carriers of leptospira and their quantum in a defined canine population will help us to know the role of dogs in the maintenance and transmission of the infection. The study will certainly be an aid in adopting appropriate preventive and control measures. Taking into consideration of these factors the present study is intended to include the following parameters

1. To assess the carrier status of leptospira in the urine and blood of dogs using darkfield microscopy, sandwich dot ELISA for leptospira antigen detection, sandwich plate ELISA for leptospira antigen detection and Polymerase chain reaction.

2. To assess the comparative efficacy of Dark field microscopy, Sandwich dot ELISA for leptospira antigen detection, Sandwich plate ELISA for leptospira antigen detection and Polymerase chain reaction for the detection of leptospira in dogs.

3. To assess the epidemiological parameters which helps in the maintenance of leptospires in canine population.

Review of Literature

2. REVIEW OF LITERATURE

2.1 HISTORY OF LEPTOSPIROSIS

The organism *Spirochaetosis icterohaemorrhagiae* was found by Kirkwood and Horning in 1923 and the disease was termed "sore mouth of dogs". In 1924 Lukes and Drivacek independently observed organisms which were morphologically identical to leptospires in the tissues of dogs which had died of Sttutgart disease. The canine strain of leptospires was studied by Klarenbeek who referred to it as *Spirochaeta ictero-uremia canis*. The organism was subsequently admitted to the classical organism *Leptospira interrogans* serotype *icterohaemorrhagiae* (Kenzy and Ringen, 1967).

In 1918 *Leptospira hebdomadis* was identified as the cause of a human condition known as seven day fever and in 1925 *Leptospira autumnalis* were shown to be the cause Japanese autumnal fever (Buxton and Fraser, 1977).

Stimson demonstrated by silver staining the presence of clumps of *spirochetes* in the kidney tubules of a patient who reportedly died of yellow fever. The *spirochetes* had hooked ends, and he named them *Spirochaeta interrogans* because of their resemblance to a question mark (Penn and Pritchard, 1990).

The etiology of leptospirosis was demonstrated independently in 1915 in Japan and Germany. In Japan, Inada and Ido detected both *spirochetes* and specific antibodies in the blood of Japanese miners with infectious jaundice, and two groups of German physicians studied German soldiers afflicted by "French disease" in the trenches of northeast France and they detected *spirochetes* in the blood of guinea pigs inoculated with the blood of infected soldiers (Levett, 2001).

It is little than 100 years since Weil; Professor of Medicine at Heidelberg (1886) whose name has been given to the disease in humans first described this disease characterized by icterus and renal failure (Sambasiva *et al.*, 2003).

Leptospirosis is a worldwide zoonosis with various names assigned to it based on the occupational groups involved like seven-day fever found commonly in Japan, Cane cutter's disease in Australia, Rice field leptospirosis in Indonesia and Fort Bragg fever, in the United States and it exists in tropical, subtropical and temperate zones in all the five inhabited continents (Sambasiva *et al.*, 2003).

2.2 BIOLOGY OF LEPTOSPIRES

Johnson and Harris (1967) opined that the pathogenic leptospires can be differentiated from the saprophytic ones by means of the failure of the former to grow at temperatures below 13°C.

The genus *Leptospira* consists of a group of spirochetal organisms, the causative agent for leptospirosis in man and animals. They are about seven to fourteen micrometer long and 0.1μ broad. The organism has got numerous elementary spirals and is characterized by hooked ends (Cruickshank *et al.*, 1975).

Johnson and Faine (1984) classified the genus *Leptospira* into two species, *L. interrogans*, comprising all pathogenic strains, and *L. biflexa*, containing the saprophytic strains isolated from the environment. Both *L. interrogans* and *L. biflexa* are divided into numerous serovars defined by agglutination after cross-absorption with homologous antigen

Leptospires are obligate aerobes. Optimal pH for growth is 7.2 to 7.6 (range 6.8 to 7.8) and optimal temperature is 28 to 32°C (Penn and Pritchard, 1990).

The slot blot method of DNA hybridization was used to study 38 strains of *Leptospira biflexa* belonging to 38 serovars. Fifteen of these serovars were placed into six DNA homology groups. The remaining 23 serovars were generally too diverse to show significant DNA relatedness either to these groups or to one another and found that genetically related organisms were antigenically dissimilar (Ramdass *et al.*, 1990)

Ramdass *et al.* (1992) examined a total of 66 serovars of potentially pathogenic Leptospira species by slot blot hybridization, and 57 of these serovars were classified in six DNA homology groups and proposed a new species, *Leptospira kirschneri.*

The phenotypic classification of leptospires has been replaced by a genotypic one, in which a number of genomospecies include all serovars of both *L. interrogans* and *L. biflexa* (Levett, 2001).

2.3 TRANSMISSION OF LEPTOSPIROSIS

Feigin and Anderson (1975) opined that many cases of human leptospirosis have been acquired from household pets, in particular from healthy dogs that have been immunized, there by creating a significant risk.

Ellis *et al.* (1976) observed that leptospires could be recovered from the milk of a cow, (suffering from leptospiral mastitis) cultured four hours after collection. They suggested that freshly drawn raw milk could constitute a limited public health hazard to farm workers.

In the carrier animals leptospires usually localize in the kidney and so infected urine is responsible for the contamination of an environment and is the common source of infection to other hosts (Buxton and Fraser, 1977).

Study on the incidence of human leptospirosis in Israel from 1970 to 1979 revealed that serovar *hardjo* infection was mostly sporadic and localized to dairy farms and cattle seemed to be the principal source of infection for man (Shenberg *et al.*, 1982).

Venkataraman and Neduncheliyan (1992a) studied an outbreak of leptospirosis in man and dog occurred during the monsoon in Madras city. *Icterohaemorrhagiae* was the most prominent serovar detected in both human beings and the animal reservoirs.

Leptospires are transmitted between animals by direct or indirect contact. Direct transmission occurs through contact with infected urine, veneral and placental transfer, bite wounds or ingestion of infected tissues whereas indirect transmission occurs through exposure of susceptible animals to contaminated water sources, soil, food or bedding (Greene *et al.*, 1998).

Murhekar *et al.* (1998) recorded that behavioral, environmental and household factors could increase the risk of exposure to leptospiral infection among human beings. Factors included exposure to contaminated environment such as exposure to stagnant water, presence of cattle or dog in the house or handling animals.

Michele *et al.* (2002) opined that epidemiological conditions of leptospire transmission could be modified by excess of rain falls or by increasing and/ or changes in rodent populations and that man could be more exposed to leptospire transmission by modifying its own occupational and leisure activities.

Sehgal *et al.* (2002) reported an outbreak of leptospirosis in Orissa and concluded that a carrier state might have existed in the animal population and the cyclone and floods changed the environment drastically making it conductive for the transmission of infection with large number of persons continuously exposed to flood waters and resulted in the outbreak.

Persons handling animals are usually at higher risk of contracting leptospirosis. This includes livestock farm workers, veterinary doctors and attendants. The prevalence of leptospirosis is higher in rural areas compared to urban population due to greater exposure to live stock (Angani *et al.*, 2003).

Barcellos *et al.* (2003) suggested that favorable ecological characteristics for leptospiral transmission include places favoring proliferation of peri -domiciliary rodents and intensive agricultural production and a higher incidence can be associated with irrigated farming.

Castellanos *et al.* (2003) noted that the major risk factors associated with leptospirosis in a rural community were flooding, having domestic animals in their home, contact with animal excreta with no protection and with a skin cut or abrasion.

Ebrahimi *et al.* (2003) stated that higher seroprevalence of leptospirosis among women in tribal areas of Central Iran could be attributed to their increased exposure to materials contaminated by urine of infected rodents and their involvement in the animal husbandry activities.

2.4 EPIDEMIOLOGY

2.4.1 World Situation

Schnurrenberger *et al.* (1962) demonstrated leptospiral agglutinins in 12 (7.3 per cent) of 164 pet dogs in Pennsylvania. Of these two had antibodies to *L. pomona*, four to *L. icterohaemorrhagiae*, three to *L. canicola* and three to antibodies to both serotypes.

Thiermann (1980) found significant titers to one or more leptospiral serotypes in 164 (37.8 per cent) of the urban stray dogs and 23 (18.7 per cent) of the 123 suburban stray dogs in Detroit. Among the urban stray dogs serotype reaction was *L. icterohaemorrhagiae* (103), *L. pomona* (7), *L. canicola* (5), *L. grippotyphosa* (12), *L. icterohaemorrhagiae* and *L. pomona* (2), *L. icterohaemorrhagiae* and *L. grippotyphosa* (4), *L. pomona* and *L. canicola* (14). In the suburban stray dogs reaction was *L. icterohaemorrhagiae* (16), *L. canicola* (3), *L. icterohaemorrhagiae* and *L. canicola* (2), *L. icterohaemorrhagiae* and *L. pomona* (2).

Yasuda *et al.* (1980) reported 21.6 per cent prevalence of leptospirosis after screening of 1428 samples of stray dogs in Brazil. Reaction was mainly against *L. canicola* (51 per cent) and *L. icterohaemorrhagiae* (25.5 per cent) with four to nine percent reacting to *L. grippotyphosa*, *L. pomona* and *L. ballum* antigens.

The results of leptospiral serology conducted on porcine sera at the central veterinary laboratory, Wey Bridge from 1971 to 1978 were analyzed. A random sample of 792 sera from 34 herds were also examined. The overall prevalence of infection was low. Serovar *copenhageni* titers were present in 7.9 per cent in diagnostic submissions (Hathaway *et al.*, 1981).

Caccciapuoti *et al.* (1982) reported a higher prevalence of leptospirosis in Somali population than that generally observed in other parts of the world. The unusually high prevalence in human leptospirosis they attribute to the contact with cattle, the main live hood of this nomadic people.

From the sera of dogs in Illinois evaluated for leptospiral antibodies, 5 per cent reaction was to leptospira serovar *bratislava*, 2.3 per cent to canicola, 1.2 per cent to *grippotyphosa* and 2.3 percent to *icterohaemorrhagiae* (Nielsen *et al.*, 1991).

Rentko and Ross (1992) reported on 17 clinical cases of leptospirosis in dogs, all of which had serological evidence of infection with *L. pomona* and *L. grippotyphosa*.

Survey conducted among cluster of leptospira patients among abattoir workers in New South Wales revealed a cluster of eight leptospirosis cases diagnosed during October and November 1998. Leptospira serovars isolated included *pomona* and *hardjo*. All the cases reported exposure to large volumes of animal urine during the course of their work (Terry *et al.*, 2000).

Michel *et al.* (2002) studied the seroprevalance of leptospirosis in small rodents and the mangoose trapped in Guadeloupe and found 48 per cent seroprevalence in myocastors, 34 to 50 per cent in the rats and 47 per cent in the mangoose, indicating that many wild animals are infected by leptospires as they secreted agglutinating antibodies.

Ebrahimi *et al.* (2003) reported that serosurvey carried out in tribal people of west central Iran showed 48.5 per cent seroreactivity to one or more leptospira serovar and noted that majority of positive serum samples reacted to *Leptospira* serovar *hardjo*. The study indicated that higher seroprevalence is related to geographical factors or traditional life systems.

2.4.2 Indian Situation

Ayyar (1932) reported the incidence of canine leptospirosis in India, while studying an outbreak of leptospiral jaundice among dogs in Madras, which was the first record of leptospirosis in India.

Pargaonker (1957) detected leptospiral infection in 10.6 per cent of the rats from the city of Hyderabad. The organisms were demonstrated by direct smear examination stained with Fontana's method or sections stained with Lavaditi's techniques.

Adinarayanan *et al.* (1960) during their investigation, found that leptospirosis due to serotypes *L. sejroe, L. medanensis and L. sarekoebing* was responsible for a disease condition among cattle population in Uttar Pradesh.

Adinarayanan and James (1980) in their three year study isolated twenty eight strains of leptospira, fourteen from pig, eight from bandicoots, one each from aborted foetus of a sheep, a goat, a rat and a mongoose and one from a piggery effluent. Serogroups identified were *Autumnalis*, *Hebdomadis*, *Javanica* and *Tarassovi*. These were the first reported isolations from animals in India.

Ratnam *et al.* (1983) attempted isolation of leptospires and demonstration of leptospiral antibodies in clinically suspected cases of human leptospirosis in Madras and found antibody titers at a low level to one or more leptospiral antigens in all the cases studied, with serovar *autumnalis* as the predominant one.

Srivastava *et al.* (1983) examined 965 sera samples from animals and man and reported 69 (7.2 per cent) samples were positive for leptospiral antibodies. They

found leptospiral antibodies in 10.1 per cent of cattle sera, 9.7 per cent of horse sera, 6.0 per cent of sheep sera, 6.0 per cent of goat sera and 5.8 per cent buffalo sera.

Cattle, sheep and goat from Haryana state were tested for Leptospira infections. Of the 1028 apparently healthy animals, 360 had antibodies to one or more serovars of *L. interrogans*. From clinical cases 92 out of 148 cattle, 23 out of 74 sheep and 7 out of 34 goats were positive for agglutinins to different serovars. In both apparently healthy and diseased animals agglutinins against *L. interrogans* serovars *wolffi, hardjo, borincana, pomona* and *szwajizak* were predominantly occurring (Batra *et al.*, 1990).

Venkataraman *et al.* (1991) studied the incidence of leptospiral jaundice among dogs in Madras and reported that of the 147 sera samples tested by Microscopic agglutination test 28, 19.04 per cent had leptospiral agglutinins and a higher incidence was noted during the post monsoon period. The study also revealed the presence of leptospiral agglutinins to *canicola* and *icterohaemorrhagiae* serovars.

A study on the seroprevalence of leptospires among dogs in Thrissur, Kerala observed *Leptospira interrogans* serovar *pomona* as the most prominent serovar and the less incriminated serovars include *canicola* and *icterohaemorrhagiae* (Indu, 1997).

Gangadhar and Rajasekhar (1998) recorded the isolation of *Leptospira javanica* from rodent species *Rattus hinton* in Karnataka, India and suggested that this serovar could be included in the battery of antigens used in the serological studies in India.

A pilot study conducted on 77 human patients during an outbreak of leptospirosis in Thane, revealed sero positivity rate of 19.48 per cent (Borwankar *et al.*, 2001).

Evaluation of the incidence of leptospirosis in Madras city during 1979 to 1993 revealed a dramatic increase in the number of cases and incidence and was noted more among males and was seen during the monsoon months. It also revealed an overall mortality of 20.8 per cent. The study revealed *L. autumnalis* to be the commonest serogroup (Muthusethupathi, 2001).

De *et al.* (2002) reported the outbreak of leptospirosis in Mumbai after a continuous heavy rainfall in the city. Out of the 102 clinically suspected cases of leptospirosis 37 were found positive.

The serological study of leptospirosis in man has been limited in India. In 1931, an extensive survey of the disease outbreak in the Andaman Islands was made and the researchers isolated*L. andamans* and *L. grippotyphosa*(Sambasiva *et al.*, 2003).

Angani *et al.* (2003) conducted a study to evaluate whether leptospirosis occurs in and around Nagpur and to determine the prevalence in various risk groups like veterinary workers, hepatitis patients and the village farmers. Out of patients in various risk groups 35 per cent tested positive for leptospira antibodies. The positivity was 32 per cent in hepatitis patients, 39.47 per cent in farm workers and the veterinary group showed a positivity of 35.71 per cent.

2.5 CLINICAL PICTURE

2.5.1 Human beings

Rathinam *et al.* (1997) opined that leptospirosis should be differentially diagnosed in human patients with acute uveitis who are having a past history of fever especially when they are from endemic areas, from lower socio economic groups and from rural areas and this can be manifested as both unilateral and bilateral panuveitis rather than as unilateral anterior uveitis.

A population based study on human leptospirosis in The Seychelles depicts head aches, lumbalgia, myalgia and conjunctival suffusions, higher erythrocyte sedimentation rates and lower platelet counts as definite indications of confirmed

leptospirosis and denotes jaundice, renal failure and pulmonary hemorrhage as the major complication occurring singly or in combination. Six deaths occurred among the 75 leptospirosis cases, reflecting a case fatality rate of 8 per cent and the cause of death was pulmonary hemorrhage confirmed by autopsy in all the cases (Yersin *et al.*, 1998).

A pilot study conducted by Borwankar *et al.* (2001) on out break of leptospirosis in Thane (Maharashtra) noted the major clinical manifestations as nausea and vomiting (53.33 per cent); hemorrhages (26.67 per cent); head and body ache (20 per cent); fever with chills and or rigors (16.67 per cent), with many patients having multiple clinical manifestations and none of the patients had jaundice or skin rashes.

Leptospirosis occurs as two clinically recognizable syndromes, the anicteric form which is a self limited illness that occurs in 85 per cent to 90 per cent of the cases and the icteric form or Weil's syndrome, is a more serious, potentially fatal syndrome and occurs in 5 per cent to 10 per cent of the cases(Sambasiva *et al.*, 2003).

Basu *et al.* (2003) noted conjunctival suffusion as pathognomonic symptom and suggested that careful examination will identify conjunctival suffusion in virtually all patients with leptospirosis.

2.5.2 Cattle

An outbreak of mastitis involving approximately 70 out of 140 cows over a two months period in United Kingdom, characterized by sudden drop in or cessation in milk production, with flaccid udders, blood tinged milk and affection of all the four quarters were studied. Common mastitis pathogens were not incriminated and leptospires belonging to the *Hebdomadis* serogroup were isolated from the milk of three out of five cows and the blood of two of those cows (Ellis, 1976).

Pearson (1980) reported that *Leptospira hardjo* appears to be predominantly confined to cattle, with the infection being transmitted from carrier cattle to

susceptible animals. It is seen associated with two clinical conditions - the milk drop syndrome and abortion

The clinical and pathological implications of leptospirosis caused by the organisms of the *hebdomadis* serogroup were tested on eight pregnant cows after their fifth month of pregnancy and was shown that they induce mild clinical manifestations to the adult host. Urinary shedding is common, and reproductive disorders and mastitis seem to be the most salient complications (Thiermann *et al.*, 1982).

Ghosh *et al.* (1989) reported a high prevalence of leptospiral serovars like *pomona* (33.33 per cent), followed by *australis* (25 per cent), *wolffi* (20.83 per cent) and *hardjo* (1.5 per cent) among cattle in organized cattle farms in North Eastern hill regions in India.

Bolin (2003) recorded that the most common cause of leptospirosis among cattle through out much of the world is infection with leptospires belonging to serovar *hardjo*. Two serologically distinguishable but genetically distinct types of serovar *hardjo* have been identified: *Leptospira interrogans* serovar *hardjo* (type *hardjoprajitino*) and *L borgpetersenii* serovar *hardjo*(type *hardjo- bovis*).

2.5.3 Sheep and Goat

Radostits *et al.* (1999) opined that leptospirosis is rare in the sheep and goat with most affected animals being found dead, apparently from septicemia and abortion may be the only clinical sign in serovar *hardjo* infection with oligolactia and agalactia observed in lactating ewes.

2.5.4 Dogs

Ayyar (1932) had described the symptoms of leptospirosis as sudden off feed, sanguineous discharge from the nostrils, passing the feces with blood and death in a few hours in Madras hounds. At the later stage icterus seen on mouth lips and under the subcutis in the region of sternum and flank. Vomiting was also

recorded in few cases. Pyrexia was recorded only in few cases where it went upto 105° F or over and dropped before death.

Stuart (1946) documented a higher incidence of leptospirosis in male dogs, more due to serovar *canicola* (47 per cent) than *icterohaemorrhagiae* (seven per cent).

Dogs of one to two years of age showed the highest percentage of leptospiral antibodies in a prevalence study in Pennsylvania (Schnurrenberger *et al.*, 1962).

Screening of a kennel of 19 dogs for a period of ten months revealed that 10 of the 19 dogs were shedding *Leptospira canicola* and none of the dogs had a history of previous illness suggestive of leptospirosis, nor were any clinical signs of renal disease detected during this study (Hubbert and Shotts, 1966).

A gradual increase in the incidence of leptospiral infection in dogs in relation to age has been reported by Thomas and Evans (1967).

A study to detect the correlation between both hepatic or renal insufficiency in dogs and leptospirosis revealed that 36.3 per cent of the animals with hepatic or renal insufficiency were leptospiral reactors with *icterohaemorrhagiae, canicola, grippotyphosa* and *bataviae* as the most incriminated serovars responsible for these conditions (Hagiwara and Rosa, 1975).

Bishop *et al.* (1979) studied chronic active hepatitis in five American Foxhounds from a kennel. *Spirochetes* were demonstrated in the liver in four of the dogs and a rising titer of *Leptospira interrogans* serovar *grippotyphosa* was found in the fifth dog. Further, a serological survey at the kennel revealed evidence of exposure of six of 13 dogs to *grippotyphosa*.

Thomas (1980) reported, a clinical case of leptospirosis due to *L. bratislava* in a ten year old German Shepard bitch which showed symptoms of increased thirst, variable appetite, weakness of the hind quarters, temperature of 106° F and

abdominal pain; progressing to jaundice, disinclination to move, vomiting and dysentery. On exploratory laprotomy bladder was full of dark coloured urine.

Navarro *et al.* (1981) found that in a group of dogs with experimentally induced leptospirosis by serovar *icterohaemorrhagiae* three dogs, from which leptospires could be isolated from blood, were not showing any apparent symptoms apart from very mild febrile reaction that occurred three days post inoculation whereas three dogs were showing only mild disease but were alert and continued to eat.

Transient leptospiremia in alert and healthy dogs were reported by Tolari *et al.* (1982). They inoculated leptospiral serovar *hardjo* in three serologically negative dogs. All the three dogs developed antibody titers to MAT with a peak in 30 days after inoculation. Transient leptospiremia with no clinical signs were noted in two dogs and no leptospires could be obtained from the urine of these dogs.

Verma (1982) in serological examination for leptospirosis among sick and healthy dogs presented to the hospitals found 47 cases to be positive. Of these 15 cases were suffering from fever, 16 of skin disease, two with bronchitis and three had hepatitis with jaindice. Rest of the seropositive dogs were clinically healthy. Higher prevalence was among the aged dogs.

Arimitsu *et al.* (1989) noticed anorexia, fever, listlessness and depression as the prominent clinical signs in 12 dogs that were infected with leptospiral serovars *icterohaemorrhagiae* and *canicola* and none had jaundice.

Rentko *et al.* (1992) had suggested that canine leptospirosis should be considered in the differential diagnosis of dogs with acute or sub acute renal failure.

Venkataraman and Nedunchelliyan (1992b) reported a case of acute fatal jaundice in a pup aged six months. The animal had a history of inappetance,

vomiting, diarrhoea and jaundice. The serological evaluation revealed it as a case of leptospiral jaundice due to serovar *canicola*.

Harkin and Gartrell (1995) noticed acute renal failure as the most common syndrome in canine leptospirosis and the most common serovars identified for this condition were *pomona, grippotyphosa* and *autumnalis*.

Interstitial nephritis due to *Leptospira interrogans* serovar *sejroe* was seen in a group of sixteen laboratory dogs, which were clinically normal. Leptospiral antigen was detected from the kidney of dogs and it was concluded that serovar *sejroe* was responsible for asymptomatic chronic renal infection (Scanziani *et al.*, 1995).

Brown *et al.* (1996) opined that *Leptospira* serovar *grippotyphosa* infection is an important problem in dogs and should be considered when evaluating a dog with renal failure.

Adamus *et al.* (1997) studied chronic hepatitis associated with leptospiral infection in sixteen Beagle dogs and found that gross lesions were confined to liver, which was firm, tan coloured and mottled. They recognized genus *Leptospira* by immunohistochemical methods from nine dogs and isolated leptospires from six dogs, where as no significant titers of antibody to leptospires were detected in these animals.

Birnbaum *et al.* (1998) opined that leptospiral serovars *pomona* and *grippotyphosa* are important pathogens capable of producing severe renal and hepatic injury in dogs.

Greene *et al.* (1998) reported leptospiral serovars *icterohaemorrhagiae, canicola, grippotyphosa, pomona* and *bratislava* as the commonly incriminated serovars in canine leptospirosis.

Observations on clinical, bacteriological and histopathological kinetics of induced leptospirosis in dogs with leptospiral serovars *autumnalis* and *canicola*

revealed that leptospiremia could be demonstrated in between post inoculation days one and five where as leptospiruria commenced in between post inoculation days five and seven and lasted through out the study period (Saravanan *et al.*, 1999).

Lieb *et al.* (1999) detected leptospiral agglutinins in 30 healthy and 20 hospitalised dogs without clinical or laboratory evidence of leptospirosis and the most incriminated serovars were *grippotyphosa*, *pomona*, *bratislava*, *australis* and *icterohaemorrhagiae*.

Adin and Cowgill (2000) recognized infection with leptospiral serovars *pomona* and *bratislava* as a cause of leptospirosis in dogs and resulted in development of acute renal failure with various degrees of azotemia.

Fehlert *et al.* (2000) observed the clinical, microbiological and pathological changes in beagle dogs infected with leptospires of the serogroup *sejroe* and found febrile temperature and haematuria as the prominent clinical manifestations.

Leptospires produce varying clinical manifestations in dogs with particular characteristics; renal, hepatic and vascular diseases are of greatest importance. Hepatic dysfunction usually was associated with the serogroups *icterohaemorrhagiae* and *pomona*, while sub acute nephritis was associated with the serogroups *canicola* and *grippotyphosa* (Noel and Kenneth, 2000).

Marshall (2001) reported that the most likely form of leptospirosis to be diagnosed in dogs in New Zealand is that due to serovar *copenhageni* and the organism produced both renal injury as well as liver damage resulting in uremia and icterus as the prominent clinical signs.

Prescott *et al.* (2002) found nonspecific clinical signs like lethargy, inappetence, dehydration, vomiting as the predominant clinical signs of leptospirosis.

Ward *et al.* (2002) evaluated the risk factors for leptospirosis among dogs in the United States and Canada and found that male dogs and dogs between four and 6.9 years of age were at significantly greater risk than the companion dogs.

Leptospira kirschneri serovar *grippotyphosa* infection was found associated with most cases of leptospirosis in dogs and the use of an effective vaccine that includes this serovar is advisable for dogs at risk of leptospirosis (Ward *et al.*, 2004)

2.5.5 Horses

Blood samples (547 no) from mares with reproductive problems in six farms of the state of Rio de Janeiro, Brazil from February 1993 until October 1996 were serologically examined for the presence of leptospiral agglutinins. A total of 235 positive reactions (42.96 per cent) were observed with predominant serovar as *icterohaemorrhagiae*, reactive on 102 (43.4 per cent) samples followed by 64 (27.3 per cent) reactions against *bratislava*, and 34 (14.47 per cent) against *pomona* (Lilenbaum, 1998).

Frazer (1999) reported a case of acute renal failure from leptospirosis in a foal. The hematological analysis and serum biochemistry revealed chances of acute renal failure and on serological evaluation the animal showed high serum titers to *Leptospira interrogans* serovar *pomona* and *bratislava*.

Faber *et al.* (2000) recorded that out of the thirty horses diagnosed to have recurrent uveitis clinically and sixteen control animals, twenty one of thirty uveitis cases and one of sixteen uveitis free cases were positive in PCR for the presence of leptospira DNA.

2.5.6 Swine

Ferguson and Power (1956) observed fever, joint stiffness, inappetance and abortion in sows experimentally infected with leptospires.

Infections in pigs were usually subclinical (or) asymptomatic, and caused abortion in late pregnancy. Occasionally metritis, icterus, anaemia, fever and meningoencephalitis (Cole, 1990) were also observed in pigs.

Firinu *et al.* (1994) reported an organized serologic survey was under taken in wild boars and free ranging pigs in Sardina, Italy. Two thousand and five hundred haemosera from wild boar and 633 from domestic swine were examined and observed that serotype *pomona* is responsible for infection among swines in extensive environment and also in wild populations in Sardina.

2.6 CARRIER STATUS

Morter *et al.* (1959) isolated *Leptospira pomona* from the urine of two healthy farm dogs and opined that farm dogs must be examined regularly for inapparent leptospirosis because of the human health hazard.

Study on the prevalence of leptospirosis in mature cattle in United States from 1986 to 1987 revealed two per cent of mature cattle as renal carriers of leptospires (Miller *et al.*, 1991).

Nielsen *et al.* (1991) found renal carriage of leptospires in dogs. He examined two dogs with previous clinical histories suggestive of leptospirosis and found antibodies to *Leptospira interrogans* serovar *bratislava* in serum from one dog and the organism was isolated from the urine of that dog.

Bal *et al.* (1994) detected long term shedding of leptospires in the urine of human beings. The presence of leptospires in urine more than one year after illness indicated that leptospires could persist in the kidneys of human beings much longer than what assumed.

Greene *et al.* (1998) stated that dogs are the persistent renal carriers of serovar *canicola* and in the survivors of the infection with this serovar renal colonization will be long term with shedding of the organism in urine for months to years.

Bolin (1999) opined that in particular regions, different leptospiral serovars are prevalent and are associated with one or more maintenance hosts and they carry and shed the organism for a long time and therefore represent the major reservoir of infection within an area.

Gangadhar *et al.* (2000) isolated *Leptospira inadai* from various rodent species in India and opined that rodents could be the natural reservoir hosts for this bacterium.

2.7 DIAGNOSIS

2.7.1 Isolation

Murphy *et al.* (1958) attempted to recover leptospires from urine of 31 dogs that were serological reactors, by intraperitoneal inoculation of hamsters. Blood sample from the heart of hamsters collected on 4, 7, 11 and 28 day post inoculation were cultured on Fletcher's semisolid medium. *Leptospira pomona* was isolated from one dog, while attempts in other 30 dogs were unsuccessful.

Venugopal *et al.* (1990) isolated leptospires from a human case by inoculation of heparinized blood into different culture media (Korthof's, Fletcher's and EMJH media) and by animal inoculation (Guinea pigs and hamsters). The isolate was identified as serogroup *autumnalis* by comparative microscopic agglutination test.

Venkataraman *et al.* (1994) demonstrated the presence of leptospires in 13 urine samples from dogs which were all seroreactors, only one isolation could be made by direct inoculation of the urine sample in EMJH semisolid medium.

Diagnosis of leptospirosis is difficult and no single diagnostic test provides optimal sensitivity or specificity and a combination of procedures including serological assays and tests to detect the presence of leptospires in tissues or body fluids is recommended (Bolin, 1996).

Gangadhar and Rajashekar (1998) isolated leptospires from different species of rodents trapped from several human dwellings and animal habitats, by culturing their kidneys in EMJH semisolid medium. Out of 500 rodents examined leptospires were isolated only from 89 of them.

Leptospiral isolation was tried to detect leptospiral material in postmortem specimens from eight human patients who died of leptospirosis and yielded only two isolates (three per cent) from 65 samples (Brown *et al.*, 2003).

A medium first devised by Ellinghausen and Maccullough and further modified by Johnson and Harris is used in a modified form known as EMJH medium for culturing leptospira strains (Gangadhar and Rajasekhar, 2001).

2.7.2 Microscopic demonstration of the organism

2.7.2.1 Darkfield microscopy

Alexander *et al.* (1957) on direct examination of urine sample under darkfield microscope detected leptospires in eight of the 76 *L. canicola* seropositive reactors and none among *L. icterohaemorrhagiae* reactors.

Leptospires could not be detected by DFM in urine of 31 seropositive dogs although leptospires were isolated from one dog (Murphy *et al.*, 1958).

Darkfield microscopy and immunoperoxidase staining test for the demonstration of antigen were compared in experimentally infected calves with serovars *pomona* and *autumnalis*. The darkfield microscopy was found better than immunoperoxidase staining both in blood and urine samples (Thillaikoothan *et al.*, 1987).

Venkataraman *et al.* (1994) detected presence of leptospires using darkfield microscopy in 2.74 per cent of the urine samples obtained from dogs with clinical symptoms suggestive of leptospirosis.

Levaditi silver stain, dark ground microscopy in wet smears, and immunofluorescence and immunoperoxidase techniques were used by Zamora *et al.* (1995) to examine kidneys of six different species of 93 wild rodents captured in the rural area of Valdivia (Chile) and recorded that leptospires were present in 40 (43.0 per cent) rodents. Levaditi's technique detected the highest number of positive samples (67.5 per cent) and the darkfield microscopy the lowest (32.5 per cent).

Chandrasekaran and Pankajalakshmi (1997) detected leptospires in the blood of three police dogs by darkfield microscopic examination, of which one dog had fever and other was asymptomatic, but failed to detect leptospires in the urine of a police dog that died out of jaundice.

Sehgal *et al.* (2001) in a study observed that DFM showing sensitivity and specificity of approximately 40 per cent in positive cases and hence it should not be used as a sole diagnostic technique in the diagnosis of leptospirosis.

Vijayachari *et al.* (2001) opined that results of DFM examination in human patients who met the gold standard criteria for leptospiral diagnosis like isolation and microscopic agglutination test and those who did not were identical indicating that the test results of DFM are not determined by the presence of leptospiral infection in the patient and hence it should not be recommended as a sole diagnostic procedure for early diagnosis of leptospirosis.

De *et al.* (2002) examined the EDTA plasma from suspected cases of leptospirosis outbreak in Mumbai using darkfield microscopy and found that 27 patients out of 102 were positive for leptospira and the study revealed a sensitivity of 26.47 per cent for DFM.

The typical motility of the leptospires in the clinical sample when correlated with clinical parameters may aid in early diagnosis. Though it is a simple method artifacts like lysed RBC'S, fibrils etc, may however be mistaken for leptospires (Sambasiva *et al.*, 2003).

Chandrasekharan and Gomathi (2004) in a study examined one hundred and eleven blood samples collected from adult as well as pediatric leptospira patients in Madurai using darkfield microscopy and DFM examination was shown to have a sensitivity of 93.3 per cent and the DFM results on paired serum samples showed persistence of leptospira in 92.9 per cent cases.

2.7.3 Polymerase chain reaction

Van Eys *et al.* (1989) first developed PCR for the detection of leptospires in urine samples of infected cattle. Urine samples were investigated using PCR assay, culture isolation, dot and quick blot hybridization. This comparative study suggested that amplification by PCR might be a valuable method for the detection of leptospires in cattle urine.

Merien *et al.* (1992) developed a sensitive assay for the detection of leptospires, based on the amplification of the leptospira *rrs* (16S) gene by PCR, and suggested that PCR assay could be used on biological samples such as CSF, urine, or blood as a diagnostic tool for cases of suspected leptospirosis.

Bal *et al.* (1994) tested urine samples from patients at different stages of Leptospirosis, to determine whether the use of PCR for detection of leptospires in urine could be a valuable alternative to culturing. Leptospires were detected in approximately 90 per cent of urine samples, and they concluded that the detection of leptospires in urine with PCR was a promising approach for early diagnosis.

Woo (1997) developed a PCR protocol based on sequence information obtained from *Leptospira* 23S rRNA genes, which has the ability to distinguish the pathogenic and saprophytic leptospiral serovars. The PCR method enabled the differentiation of the 59 strains of the 23 serogroups of *Leptospira interrogans* from the 8 strains of 6 serogroups of *Leptospira biflexa*.

An IS 1500 based PCR assay was developed for identification of *Leptospira interrogans sensulato* serovars (Zuerner and Bolin, 1997). They concluded that this

assay was selective enough to be used for typing leptospiral serovars from clinical material, and for attaining leptospiral typing without isolation of bacteria in pure culture.

Letocart *et al.* (1997) described the construction of leptospira species specific probes produced by using Arbitrarily Primed PCR, in order to identify three main pathogenic species of *leptospira*, namely *Leptospira interrogans*, *Leptospira borgpetersenii*, *Leptospira kirschneri*.

Samples of cerebrospinal fluid from 103 patients with aseptic meningitis were tested by PCR for detection of leptospires and the results were compared with MAT and ELISA . 39.8 per cent positivity was observed with PCR and 8.74 per cent and 3.88 per cent were positive by MAT and Ig M ELISA respectively (Romero, 1998).

Heinemann *et al.* (1999) compared the PCR, culture/isolation and serology to detect leptospiral infection in bovine semen. Eighty per cent of semen samples were found to be positive by PCR and leptospires could not be isolated from any of the samples examined. Polymerase chain reaction was found to be a method of great potential for the detection of leptospires.

Faber *et al.* (2000) detected Leptospira DNA in the aqueous humor of horses with naturally acquired recurrent uveitis, by polymerase chain reaction. Serological results did not correlate well with the presence of organism in the aqueous humor and indicated the PCR assay as a more reliable tool for detecting leptospirosis in equine recurrent uveitis.

Senthilkumar *et al.* (2001) used PCR for the detection of leptospires in clinical samples like, blood, urine, CSF and milk and compared the results with those of DFM and concluded that PCR assay was highly sensitive and specific for detection of leptospira infection.

Ramdass *et al.* (2002) investigated the use of Arbitrarily Primed polymerase chain reaction for typing leptospiral serovars and concluded that this technique offers great potential for simple and rapid identification of leptospires at serovar level, which could be useful in molecular epidemiological studies of leptospirosis.

Harkin *et al.* (2003a) employed polymerase chain reaction as a diagnostic tool for leptospirosis in dogs and compared the results with those of microagglutination test. Positive PCR test results prior to seroconversion could be valuable in establishing an early diagnosis.

Harkin *et al.* (2003b) in their study on prevalence of urinary shedding of leptospires in dogs compared PCR assay, bacteriologic culture of urine and serological testing. Irrespective of the health status, urinary shedding of leptospires was observed in 8.2 per cent of dogs and concluded the serological testing as a poor predictor of urinary shedding.

Shukla *et al.* (2003) described 16S rRNA PCR for differentiation of pathogenic and non pathogenic leptospira isolates.

2.7.4 Serological tests

2.7.4.1 Microscopic agglutination test (MAT)

Muthusethupathi (2001) reported that MAT is a test that can identify the serovars of leptospira, which is of epidemiological importance and the main disadvantage is that the titers rise late in the course of infection

Venkataraman *et al.* (1994) studied the leptospiral agglutinins in dogs in Madras city and of the 474 sera samples tested 49 (10.34 per cent) had leptospiral antibodies with predominant reaction to serovar *canicola* followed by *icterohaemorrhagiae.*

Weekes *et al.* (1997) evaluated the seroprevalence of leptospirosis in healthy as well as diseased dogs using microscopic agglutination test and found that *autumnalis* and *icterohaemorrhagiae* as the prominent serovars in respective groups.

Anti leptospiral agglutinins were detected in the sera of feral pigs using microscopic agglutination test. Seropositivity of 20 per cent was detected with majority of serological reactors to serovar *pomona* (Mason *et al.*, 1998).

Myers (1980) examined sera from 143 stray dogs by microscopic agglutination test. A total of 73 dogs had contact with leptospires and the predominant agglutinins were to serovar *canicola* and to a lesser extend to serovars *pyrogenes* and *autumnalis*.

MAT was used for serological study of leptospiral infections among dairy cows in farms with sub optimal reproductive efficiency in Spain and serovars *bratislava* and *grippotyphosa* were detected as the most prevalent serovars (Guitian, *et al.*, 2001).

Sambasiva *et al.* (2003) reported that in human beings MAT titer of 400 to 800 or more, or a four fold rise in titer between the two tests is diagnostic when combined with a clinical illness compatible with leptospirosis.

Talpada *et al.* (2003) examined 1193 Texas slaughter house cattle serum samples for anti leptospiral agglutinins by MAT and found that 262 (22 per cent) reacted with serovar *pomona* and 179 (15 per cent) with serovar *hardjo*.

2.7.4. 2 Macroscopic agglutination test

Srivastava (1990) studied the efficacy of the slide agglutination test (SAT) for detecting leptospirosis in various animal species and man. He opined that SAT alone might not be useful for detecting leptospirosis in cattle and sheep, as it poorly correlated with MAT and concluded that low MAT titres and a strong SAT reaction in animals could be suggestive of a recent infection.

Evaluation of a rapid slide agglutination test using formalized antigenic suspensions of *Leptospira interrogans* serovar *canicola* and *copenhageni* for leptospira antibodies in canine serum revealed it as a simple, less expensive test, which can be performed for the screening of large number of serum samples and when compared to microscopic agglutination test, presented good sensitivity and specificity rates (Lilenbaum *et al.*, 2002).

2.7.4. 3 Latex agglutination test

A rapid semi-quantitative latex agglutination test (LAT) for the detection of leptospiral antibodies in serum samples of man and animals was standardized (Ramadass *et al.*, 1999). The efficacy of the LAT was compared with the plate assay. The rapidity, simplicity and economics of the LAT were found to fulfill the requirements of a screening test, for leptospiral antibodies.

A newly developed latex agglutination assay for the detection of genus specific *Leptospira* antibodies in human sera was evaluated. The mean overall sensitivity was 82.3 per cent and the mean overall specificity was 94.6 per cent. The assay is easy to perform and does not require special skills or equipment (Smits, 2000).

The sensitivity, specificity, positive and negative predictive values of eight rapid screening tests for acute leptospirosis in Hawaii found that Latex agglutination test was found to be highly sensitive, but the specificity and positive predictive value of this test were unacceptably low (Effler, 2002).

2.7.4.4 Indirect Haemagglutination Assay

Srivastava *et al.* (1985) standardized an indirect haemagglutination (IHA) test for the detection of antibodies against leptospira organisms in different animal species. Indirect haemagglutination was found to be quite simple and sensitive.

Levett and Whittington (1998) evaluated a commercially available indirect haemagglutination assay (IHA) with multiple serum specimens from 107 patients for

leptospirosis. Indirect haemagglutination detected both IgM and IgG classes of antibodies in human sera, the sensitivity and specificity of IHA for the detection of acute leptospirosis were 100 and 94 per cent respectively. Investigation of serum specimens from 27 dogs showed concordance between IHA and an IgM ELISA. They concluded that the performance of IHA was simple, and required no specialized equipment.

Comparitive serological assay of leptospirosis in Hawaii from 1992 to 1997 among human beings using indirect haemagglutination and microscopic agglutination test revealed indirect haemagglutination as a less sensitive test for the serodiagnosis of acute leptospirosis (Effler *et al.*, 2000).

Evaluation of four commercially available rapid serological tests for diagnosis of leptospirosis found that indirect haemagglutination assay shows a wide range of sensitivities with sensitivities 38.5 per cent for acute sera and 68.2 per cent for convalescent sera (Bajani *et al.*, 2003).

2.7.4.5 Immunoperoxidase staining

Terpstra *et al.* (1983) demonstrated leptospires in blood and urine by immunoperoxidase staining method.

Ellis *et al.* (1983) described the development and testing of a peroxidase-labelled antibody method for the detection of leptospires in tissues and smears of infected fluids, that could detect leptospires in formalin fixed paraffin embedded kidney sections and found good correlation with cultural results.

Immunoperoxidase test was evaluated for the diagnosis of leptospirosis by demonstration of leptospires in blood and urine of experimentally infected bull calves (Thillaikoothan *et a*l., 1987).

Horseradish peroxidase enzyme conjugated to immunoglobulins or antiglobulins may be used to identify specific antigens in tissue sections and in the

treated tissue sections the bound antibody is detected as brown deposits, which can be visualized by conventional light microscopy (Tizard, 1996).

Perriera *et al.* (1997) examined kidneys of golden hamsters experimentally infected with *Leptospira interrogans* serovar *canicola* using avidin – biotin immunoperoxidase staining and observed typical, well defined morphologic leptospires near to blood vessels, within inflammatory infiltrates and intraluminal in proximal and distal parts of the nephron.

2.7.4.6 Immunofluorescence.

Immunofluorescence could be able to detect leptospires in 41 kidney tissue samples out of 5142 samples collected from mature cattle in 49 states of United States of America and the test was effective in detecting leptospires from sero negative and culture negative samples (Miller *et al.*, 1991).

Dhaliwal *et al.* (1996) examined cervico-vaginal mucus and post calving discharges from 163 bulling cows naturally infected with *Leptospira interrogans* serovar *hardjo* for the detection of antigen using direct fluorescent antibody test. A positive result for antigen was observed in the cervico vaginal mucus of six animals and none were positive in the examination of post calving discharges.

Antibody directed against particular bacteria or virus is labeled with Fluorescein isothiocyanate and can be used for the specific detection of antigen of our interest. The bound antigen antibody complex can be visualized using a fluorescent microscope (Tizard, 1996).

Greene *et al.* (1998) opined that fluorescent antibody techniques could be used to identify leptospires in tissue imprints of liver and kidney in dogs. It can also be used as a screening method to identify animals shedding leptospires in urine.

Fluorescent antibody test was performed on urine samples of a foal suspected of acute renal failure and it confirmed the presence of organism, indicating that the foal was actively shedding the organism (Frazer, 1999).

Direct immunofluorescence detected leptospires in autopsy material of 2 out of 18 human patients (11 per cent) died due to leptospirosis and it showed intermediate sensitivity for detecting leptospires when compared with leptospiral culture and PCR analysis (Brown *et al.*, 2003).

2.7.4.7 Enzyme Linked Immuno Sorbant Assay (ELISA)

2.7.4.7.1 Antigen detection

A dipstick enzyme linked immunosorbent assay was standardized to detect the leptospiral antigen from human urine sample. The test was applied to 30 suspected human urine samples and got a positivity on 8 samples (26.6 per cent) and the results were matching with the microscopic agglutination test in the sera of corresponding cases (Sureshbabu *et al.*, 1996).

Saengjaruk *et al.* (2002) evaluated the diagnosis of human leptospirosis by monoclonal antibody based antigen detection in urine. They stated that monoclonal antibody based dot –ELISA has a high potential for rapid, sensitive and specific diagnosis of leptospirosis at a low cost.

2.7.4.7.2 Antibody detection

Thillaikoothan *et al.* (1987) employed Microscopic agglutination test and ELISA for the antibody detection in bull calves experimentally infected with leptospiral serovars *pomona* and *autumnalis*. The MAT detected early serum antibodies than the ELISA, the reason may be due to the use of IgG fraction in the conjugate.

Gussenhoven *et al.* (1997) developed an easy to perform dipstick method for the detection of leptospira specific IgM antibodies in human serum samples and observed a high degree of concordance between the results of the dipstick assay and IgM-ELISA.

Smits *et al.* (1999) performed a multicentre evaluation of a dipstick assay for the detection of leptospira-specific immunoglobulin M (IgM) antibodies. The

sensitivity of the dipstick assay increased from 60.1 per cent for acute phase serum samples to 87.4 per cent for convalescent phase samples. The specificities for these two groups of samples were 94.1 and 92.7 per cent respectively. The results were concordant with the results of ELISA for the detection of specific IgM antibodies.

Srivastava and Tiwari (1999) reported an IgG based Dot-ELISA for the diagnosis of leptospirosis in goats and found that Dot-ELISA could be a better replacement for MAT.

Matsuo *et al.* (2000) utilized exocellular mannan from *Rhodotorula glutinins* as an immuno reactive antigen in serological diagnosis of leptospirosis and found it to be useful as an antigen in ELISA as it specifically cross reacted with the anti-leptospiral antibodies.

Sekhar *et al.* (2000) compared two commercially available ELISA's with microscopic agglutination test for the serodiagnosis of leptospirosis and found that the INDX Dip - S – Ticks dot assay is a practical alternative to Microscopic agglutination test.

The diagnostic utility of recombinant antigens in ELISA for serodiagnosis of leptospirosis was evaluated by Flannery *et al.*,(2001). The sensitivity of recombinant Lip 32 IgG- ELISA was found to be 56 and 94 per cent in acute and convalescent phases respectively. They concluded that recombinant Lip L32 could be a useful antigen for the serodiagnosis of leptospirosis.

Relative superiority of IgM + IgG combined conjugate ELISA for the detection of leptospiral antibodies was studied and indicated that this test could be well utilized in seroprevalence studies. (Natarajaseenivasan and Ratnam, 2001).

Materials and Methods

3. MATERIALS AND METHODS

The research work was carried out at the Department of Veterinary Epidemiology and Preventive Medicine college of Veterinary and Animal Sciences, Mannuthy during the period June 2002 – May 2004.

3.1 MATERIALS

3.1.1Glassware, plasticware and Chemicals

All glassware used was of Borosil brand. Plasticware used were of Tarson brand and chemicals were of analytical or guaranteed grade.

3.1.1.1 Sterilisation of Glassware and Plasticware

New glassware were kept overnight in potassium chromate solution (Potassium chromate crystals – 80 mg, Concentrated sulphuric acid – 250 ml, Distilled water – 750 ml).

The glassware was then washed twice in ordinary tap water and also in distilled water. It was dried and sterilized by keeping in hot air oven at 160° C for one hour after plugging with non absorbent cotton.

Plasticware were sterilized by autoclaving at 121° C for 15 minutes at 15 pounds pressure.

3.1.2 Samples

Blood and Urine samples (210 no.) collected from both healthy and diseased dogs brought to University Veterinary Hospitals, Kokkalai and Mannuthy formed the materials of study.

3.1.3 Buffers and Reagents

3.1.3.1 Phosphate Buffered saline (PBS) Stock Solution (10x)

Sodium chloride	80 g
Potassium chloride	2 g
Disodium hydrogen phosphate	11.32 g
Potassium dihydrogen phosphate	2 g
Distilled water	1000 ml

The pH was adjusted to 7.4 by 1N NaOH and sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure. The stock solution was diluted to 1x before use.

3.1.3.2 Buffered Anticoagulant

Sodium oxalate	1.0 g
Phosphate buffered saline	100 ml

Buffered anticoagulant solution was sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure.

3.1.4 Sandwich Plate ELISA kit for Leptospira antigen detection.

Sandwich Plate ELISA kit for Leptospira antigen detection was obtained from The Division of Microbiology, Defence Research and Development Establishment (DRDE), Gwalior (Fig. 1).

3.1.4.1 Components

Test kit for testing 36 – 94 samples

1. *Micro titre ELISA plate 1
2. 12 tip blood collection combs 8

Fig.1.SANDWICH PLATE ELISA KIT FOR ANTIGEN DETECTION

COMPONENTS

1.Dilution buffer
2.Monoclonal antibody
3.Conjugate
4.Substrate buffer
5.Substrate powder
6.Substrate solution dropper vial
7.Stop solution
8.Wash buffer
9.Microtitre ELISA plate
10.Metal spoon

3. Dilution buffer bottle	4
4. *Conjugate bottle	2
5. *Monoclonal antibody bottle	2
6. Wash buffer	3
7. *Substrate powder vial	2
8. *Substrate buffer bottles	3
9. Substrate solution dropper vials	3
10. Stop solution bottle	1
11. Small metal spoon	1
12. Plastic syringe	1
13. Safety Pin	1
14. Lead Pencil	1
15. Blade	1

* To be stored at 4°C

3.1.5 Sandwich dot ELISA kit for Leptospira antigen detection.

Sandwich dot ELISA kit for Leptospira antigen detection was obtained from The Division of Microbiology, Defence Research and Development Establishment, Gwalior (Fig. 2).

3.1.5.1 Components

Test kit for testing 80 samples.

1. *Vial with NC cap	80
2. Dilution buffer bottle	4
3. *Conjugate bottle	2
4. *Monoclonal antibody bottle	2
5. Wash buffer	3
6. *Substrate powder vial	2
7. Small metal spoon	1

Fig.2.SANDWICH DOT ELISA KIT FOR LEPTOSPIRA ANTIGEN DETECTION

COMPONENETS.

1. DILUTION BUFFER
2. MONOCLONAL ANTIBODY
3. CONJUGATE
4. SUBSTRATE BUFFER
5. SUBSTRATE POWDER
6. VIAL WITH NC CAP
7. WASH BUFFER
8. METAL SPOON

8.	Plastic syringe	1
9.	Safety Pin	1
10.	Lead Pencil	1
11.	Blade	1

* To be stored at 4°C

3.1.6 Polymerase Chain Reaction

3.1.6.1 Tris EDTA Borate Buffer (TBE) pH 8.2

Tris base	90mM
Boric acid	90mM
EDTA	2mM
pH	8.4

3.1.6.2 Ethidium Bromide Stock Solution

Ethidium bromide	10 mg
Triple distilled water	1 ml

3.1.6.3 Agarose Gel

Loading gel was prepared by adding 0.8 per cent agarose in tris EDTA borate buffer and was boiled. Five micro liters of Ethidium Bromide stock solution was added in the gel before it solidifies.

3.1.6.4 Gel loading buffer (6 x)

Bromophenol blue	0.25 per cent
Xylene cyanol	0.25 per cent
Sucrose	40 per cent (w/v) in water

3.1.6.5 Preparation of DNA from clinical samples

a. QIA, DNA extraction kit -

Silica gel membrane technology was used for rapid and efficient purification of total cellular DNA.

b. Enzymatic lysis buffer

It contains 20mM Tris – Cl pH 8.0, 2mM EDTA, 1.2 per cent Triton X – 100 and 20mg/ml lysozyme.

c. Proteinase – K

d. AL Buffer

e. Ethanol - *Alcohol*, 70 per cent

Absolute alcohol 70 ml was added with 30 ml of triple glass distilled water, and stored at 4°C.

f. Wash buffer 1 (AW1)

g. Wash buffer 2 (AW2)

h. Elution buffer (AE buffer)

3.1.6.6 PCR amplification

a. Primers

Primer sequences for 16 S rRNA (Hookey 1992) were synthesized from M/s Genetix, New Delhi.

P1 – 5' CGCTGGCGGCGCGTCTTAAA3' 20 mer

P2 – 5' TTCACCGCTACACCTGGAA3' 19 mer

b. PCR Reaction Buffer (10x)

This includes 500 mM KCl, and 100 mM Tris hydrochloride

c. Magnesium Chloride

Magnesium chloride with strength of 25mM.

d. *Taq* DNA Polymerase

The *Taq* DNA polymerase enzyme with a concentration of 3 U/µl

e. Deoxy Nucleotide Triphosphates

Deoxy nucleotide triphosphates (dNTP mix) 10mM (2.5 mM of each dGTP/ dCTP/ dATP/ dTTP).

3.2 METHODS

3.2.1 Screening of dogs for leptospirosis

To detect the presence of leptospirosis and to assess the carrier status in dogs for leptospirosis, 210 dogs were screened. This included 101 healthy dogs and 109 diseased dogs. The detailed clinical observations, history of symptoms suggestive of leptospirosis and the vaccination status of dogs screened were collected as per the proforma attached (Appendix 1).

3.2.2 Collection of samples

Blood and urine samples were collected from 210 dogs, both healthy and diseased, presented to the University Veterinary Hospitals Kokkalai and Mannuthy. The samples were examined for leptospires by means of dark field microscopy

(DFM), Sandwich Dot ELISA for leptospira antigen detection and sandwich plate ELISA for leptospira antigen detection. Both serum and urine samples were preserved at - 20° C until use and Polymerase chain reaction was performed on all the samples to detect the pathogenic leptospires.

3.2.2 .1 Blood and serum

Blood samples were collected aseptically from 210 dogs by vein puncture. About three milliliter of blood samples was collected in sterile vials containing sodium oxalate anticoagulant. About four milliliter of blood was also collected in sterile syringe (five ml syringes) and the sample was kept undisturbed in the slanting position for the serum to form. Serum was collected in sterile eppendorf tubes.

3.2.2.2 Urine

Urine was collected aseptically from the 210 dogs by catheterization. Infant feeding tubes size five and six were used for the same. About five milliliter of urine was collected onto equal quantity of sterile Phosphate buffered saline, pH 7.4 (Cruickshank *et al.*, 1975) in sterile vials.

3.2.2.3 Preservation of samples

Both urine and serum samples were preserved for the purpose of performing Sandwich dot ELISA, Sandwich plate ELISA and Polymerase chain reaction to find out the pathogenic leptospires. About two milliliter of serum and three milliliter of urine were preserved in sterile eppendorf tubes by adding one per cent merthiolate solution @ 20 micro liters per ml of sample and the samples were kept at - 20° C until use.

3.2.3 Dark field microscopic examination

3.2.3.1 Blood

Two milliliter of blood samples collected in buffered anticoagulant was processed for microscopic examination. The blood was centrifuged at 1000 rpm for 15 min, and the plasma was separated. A drop of plasma was placed on a clean, grease free glass slide and applied a cover slip. This wet mount preparation was examined under low (10x) and high (45x) power objective of the dark field microscope. Utmost care was taken to examine as many microscopic fields as possible with a minimum of 100 high power fields. The plasma was centrifuged at 10000 rpm for 15 min and the sediment was examined on the same way.

3.2.3.2 Urine

Five milliliter of urine samples collected in sterile PBS was centrifuged at 3000 rpm for 10 min. A drop of sediment was placed on a clean, grease free glass slide, and applied a cover slip. The slide was examined under low (10x) and high (45x) power objective of the dark field microscope to demonstrate the presence of leptospires. While examining utmost care was taken to observe as many microscopic fields as possible with a minimum of 100 high power fields.

3.2.4 Sandwich Dot ELISA for Leptospira antigen detection

Sandwich Dot ELISA for Leptospira antigen detection was done on the samples – Blood and urine samples as per the protocol of DRDE, Gwalior.

1. To the vial cap containing the Nitrocellulose paper about 80µl of dilution buffer was added. To this 20µl of the sample was added and incubated for half an hour.
2. After incubation, added a few drops of wash buffer onto the caps containing NC membrane and poured off the contents. Took about one milliliter of wash buffer in the vial and then closed with the NC cap and then shook the vial vigorously for one minute. Poured of the wash buffer and repeated the

washing for two times. After the final wash the NC cap was made dry by gentle tapping.

3. To the NC membrane added two drops of monoclonal antibody and incubated for a period of 30 min.
4. After incubation repeated the washing step as said earlier and made the NC cap dry.
5. To the NC membrane two drops of conjugate was added and incubated for 30 min.
6. After incubation repeated the washing step as said earlier and made the NC cap dry.
7. Took about one milliliter of substrate buffer in the vial and to this half spoon full of substrate powder was added. The vial was closed with the NC cap and the vial was shaken vigorously for two to three minutes.
8. After this looked for the colour reaction in the NC membranes. The positive and negative results were observed as characteristic brown dots in the nitrocellulose membrane. (Fig. 3)

3.2.5 Sandwich plate ELISA for Leptospira antigen detection

Sandwich plate ELISA for Leptospira antigen detection was done on the samples – Blood and urine samples as per the protocol of DRDE, Gwalior.

1. Removed the adhesive tapes over the well to be used.
2. 90µl of dilution buffer and 10 µl of test sample (Urine/ blood) was added onto the wells and incubated for 30 min.. (To the last two wells of the plate known positive and known negative samples were added).
3. After incubation poured off the contents in the well and the wells were washed three times using wash buffer for a minute by gentle shaking of the plate. Stack dried the wells.
4. To each well three drops of Monoclonal antibody was added and incubated for a period of 30 min.

5. After incubation repeated the washing step as before and stack dried the wells.
6. To each well three drops of conjugate was added and incubated for a period of 45 min.
7. After incubation repeated the washing step five times and stack dried the wells.
8. Substrate buffer was prepared in the substrate vial by adding half small spoon of substrate powder to the substrate buffer, taken up to the mark shown in the vial.
9. Added substrate buffer onto each well and mixed gently by shaking and waited for the colour reaction in the well loaded with known positive sample.
10. Added stop solution to each well and the results were read visually in comparison with the colour reaction in the well loaded with known positive sample.
11. The positive results were observed as characteristic golden yellow colour as seen in the well loaded with the known positive sample. (Fig. 4).

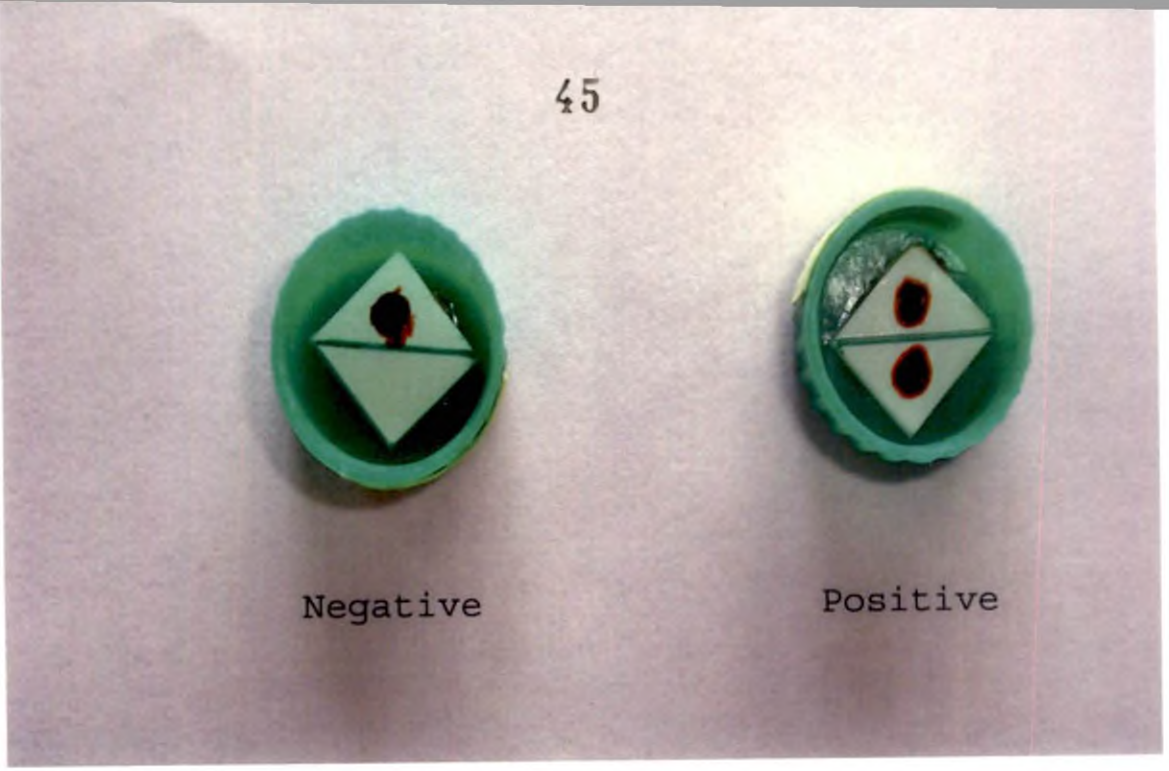

Fig. 3.
SANDWICH DOT ELISA FOT LEPTOSPIRA ANTIGEN DETECTION

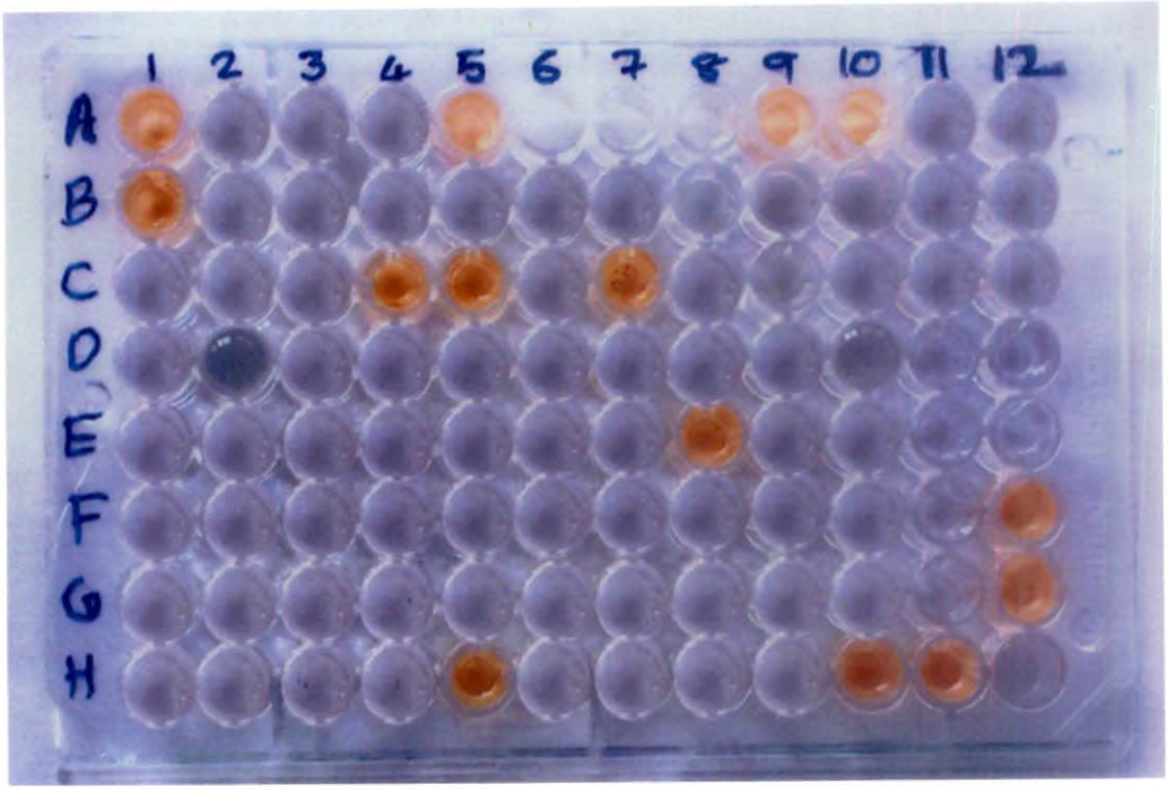

Fig. 4.
SANDWICH PLATE ELISA FOR LEPTOSPIRA ANTIGEN DETECTION

Test sera samples	A1 to H10
Positive control	H11
Negative control	H12
Positive sera samples	A1,A5,A9,A10
	B1,C4,C5,C7,E8,
	F12,G12,H5,H10

3.2.6 Polymerase Chain reaction

The clinical samples, serum and urine samples were examined by means of polymerase chain reaction for detecting pathogenic leptospires (Hookey, 1992).

3.2.6.1 Preparation of DNA from clinical samples

1. The bacteria in the samples were pelleted by centrifugation for 10 min. at 10,000 rpm at 4°C.
2. The pellet was suspended in 180 µl of enzymatic lysis buffer and incubated for 30 min. at 37°C
3. 25µl of Proteinase K and 200µl of buffer AL was added and mixed by vortexing. Incubated the mixture at 70°C for 30 min.
4. 200µl of ethanol was added and mixed by vortexing.
5. Pipetted out the mixture into Dneasy mini column and centrifuged at 8,000 rpm for one minute and the flow through were discarded.
6. 500µl of wash buffer 1 (AW1) was added into the column and centrifuged at 8,000 rpm for a minute and the flow through was discarded.
7. 500µl of wash buffer 2 (AW2) was added into the column and centrifuged at 10,000 rpm for three minutes and the flow through was discarded.
8. The column was then placed in two milliliter tube and 200µl of Elution buffer (AE buffer) was added
9. Incubated at room temperature for one minute and centrifuged at 8000 rpm to elute the DNA.

3.2.6.2 PCR Amplification

To set up a PCR reaction, 20 µl of master mix was prepared as

10 X PCR buffer	2.5µl	(1X)
25Mm $MgCl_2$	2.5µl	(4mM)

dNTPs (10mM each)	0.5µl	(200 µM)
Primer P1	1.5µl	0.1 – 0.5 µM
Primer P2	1.5µl	0.1 – 0.5 µM
Taq DNA Polymerase	0.3µl	1.5 units
Distilled water	11.2 µl	

To each PCR tube 20µl of master mix and five microliter of template DNA were added. One negative control without template DNA and a standard saprophytic leptospiral DNA was also included. The tubes were spun briefly and placed in the thermal cycler.

3.2.6.2a PCR amplification conditions

The reaction is carried out in thermal cycler under following PCR amplification conditions.

Step – 1		
Initial denaturation	94°C for 5 minutes	1 cycle
Step – 2		
Denaturation	94°C for 1 minute	
Annealing	55°C for 1 minute	35 cycles
Extension	72°C for 1 minute	
Step – 3		
Final extension	72°C for 10 minutes	

Amplification of DNA was performed in Eppendorf mastercycler.

3.2.6.3 Detection of PCR products

3.2.6.3a Submarine Agarose gel Electrophoresis

The Agarose gel was prepared by adding 0.8 per cent agarose in tris EDTA borate buffer and boiled. Five microliter of ethidium bromide was added into the gel before it solidified.

A clean, dry gel platform was selected and the sides were sealed using good quality adhesive tapes. The suitable combs intended for preparation of wells are placed in the platform. Agarose was poured into the plat form so as to make a gel of five millimeter thickness. Once the gel gets solidified, the combs were taken out and then the wells can be appreciated.

The samples were loaded along with the DNA markers and the gel was run at 50 V for two hours. The amplification was observed under transilluminator (Bio Rad, USA) and was documented using gel documentation system (Gel Doc, Sony, Japan).

Appearance of a band at 630 bp when compared with the DNA ladder is taken as positive (Fig. 5).

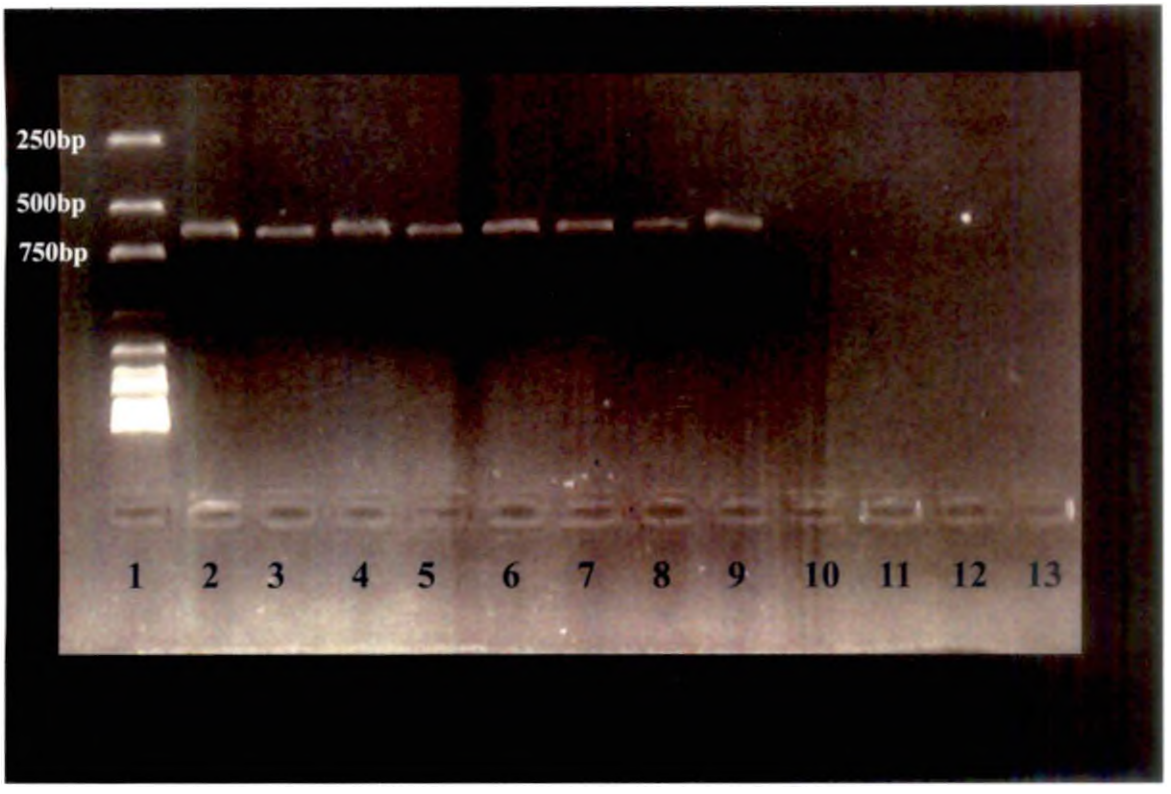

Fig. 5. 16S rRNA PCR for leptospira

1. DNA Ladder- 250bp
2. Positive control
3-9 Positive samples
10. Negative control
11-13. Negative samples

Results

4. RESULTS

4.1 SCREENING OF DOGS FOR LEPTOSPIROSIS

Blood and urine sample were collected from 210 dogs brought to the University Veterinary hospitals at Kokkalai and Mannuthy for screening leptospirosis. This included 101 healthy animals and 109 diseased animals. The detailed clinical observation and history were recorded as per the proforma.

4.1.1 Screening of healthy dogs

The result of screening of healthy dogs for leptospires using Dark field microscopy, Sandwich plate ELISA for *Leptospira* antigen detection, Sandwich dot ELISA for *Leptospira* antigen detection and Polymerase chain reaction are presented in table (1). Out of the 101 healthy dogs screened for leptospires 18 (17.82 per cent) animals were found positive. Leptospiremia alone could be detected in four (3.96 per cent) healthy animals, where as leptospiruria alone was detected in 12 (11.88 per cent) healthy dogs. Two (1.98 per cent) dogs were found to have both leptospiremia and leptospiruria (Fig.6).

4.1.2 Screening of diseased dogs

The dogs presented to the University Veterinary hospitals at Kokkalai and Mannuthy with any of the symptoms like fever, anorexia, vomiting, jaundice, haematuria or haematemesis were screened for *Leptospira* using Dark field microscopy, Sandwich plate ELISA for *Leptospira* antigen detection, Sandwich dot ELISA for *Leptospira* antigen detection and Polymerase chain reaction and the results are presented in table (1). Twenty four (22.02 per cent) out of the 109 diseased dogs presented were found positive for leptospirosis. Leptospiremia could be detected 16 (14.68 per

cent) diseased dogs, leptospiruria in four (3.67 per cent) and both Leptospiremia and leptospiruria was found in four (3.67 per cent) diseased dogs (Fig.6).

4.2 CLINICAL MANIFESTATIONS OF LEPTOSPIROSIS IN DOGS

Clinical manifestations of the leptospirosis in dogs presented to the University Veterinary hospitals Mannuthy and Kokkalai were noted. The prominent clinical manifestations of canine leptospirosis revealed by this study included anorexia, fever, vomiting, jaundice and haematuria. Anorexia was present in 18 (75 per cent) diseased animals, which turned out positive for leptospirosis. Fever was present in 18 (75 per cent) cases, vomiting was present in eight (33.33 per cent) dogs, jaundice in two (8.33 per cent), haematuria in two cases (8.33 per cent) and haemoglobinuria was present in one (4.17 per cent) case. (Table 2 & Fig. 7).

4.3VACCINATION STATUS IN DOGS SCREENED FOR LEPTOSPIROSIS

Vaccination status of 210 dogs screened for leptospirosis was collected. Out of the total 42 dogs found positive for leptospirosis, proper vaccination status was present only for 15 (35.71 per cent) animals. This included seven (16.67 per cent) healthy and eight (19.05 per cent) diseased dogs. Fifteen (35.71 per cent) of the leptospira positive animals, which included nine (21.42 per cent) healthy and six (14.29 per cent) diseased dogs, were not at all vaccinated where as eight (19.05 per cent) animals , two (4.76 per cent) healthy and six (14.29 per cent) diseased were having an improper vaccination status, with vaccines taken in their early life with delay in the annual booster vaccinations. No history regarding vaccination status was available with four (9.52 per cent) diseased dogs presented (Table 3).

Table 1. Leptospira screening in healthy and diseased dogs

Source	Total No. Tested	Leptospiremia		Leptospiruria		Both		Total	
		No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Healthy dogs	101	4	3.96	12	11.88	2	1.98	18	17.82
Diseased dogs	109	16	14.68	4	3.67	4	3.67	24	22.02
Total	210	20	9.52	16	7.61	6	2.8	42	20

Table. 2 Clinical manifestations in dogs positive for leptospirosis

Clinical signs	No:	%
Fever	18	75
Anorexia	18	75
Vomiting	8	33.33
Jaundice	2	8.3
Haematuria	2	8.3
Haemoglobinuria	1	4.17

Table. 3 Vaccination status in dogs positive for leptospirosis

Total no. of dogs screened		No. of dogs positive for leptospirosis	With proper Vaccination		Improper Vaccination		No Vaccination		History not available	
			No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Healthy	101	18	7	16.67	2	4.76	9	21.42	-	-
Diseased	109	24	8	19.04	6	14.29	6	14.29	4	9.52
Total	210	42	15	35.71	8	19.05	15	35.71	4	9.52

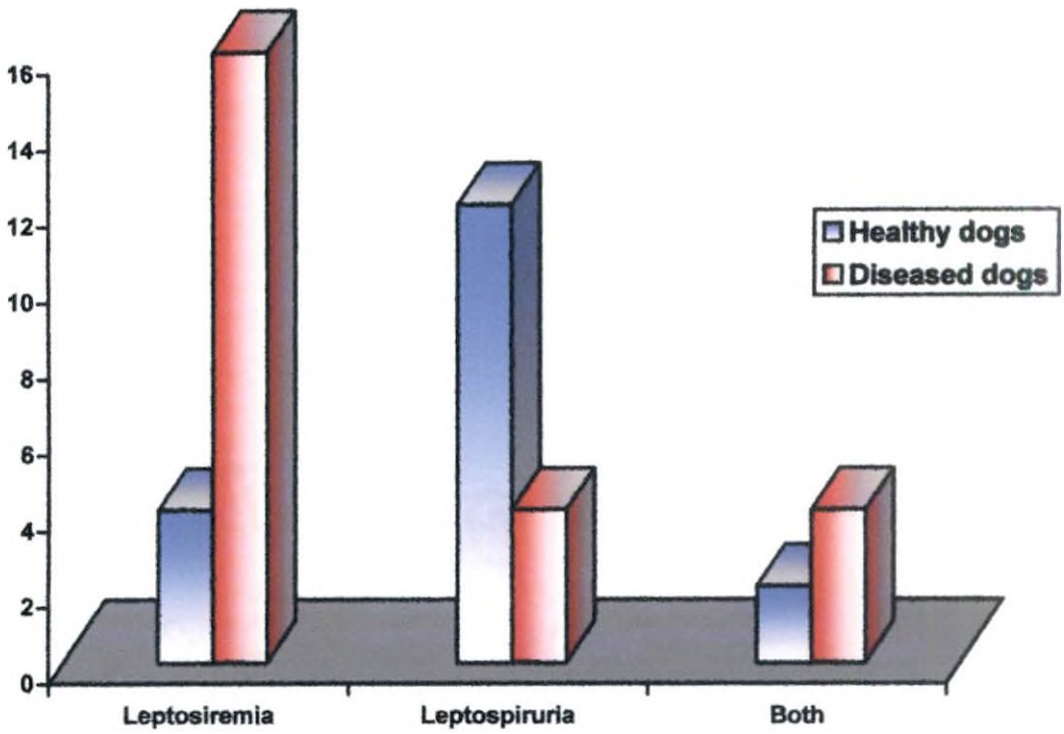

Fig. 6 Leptospira screening in dogs

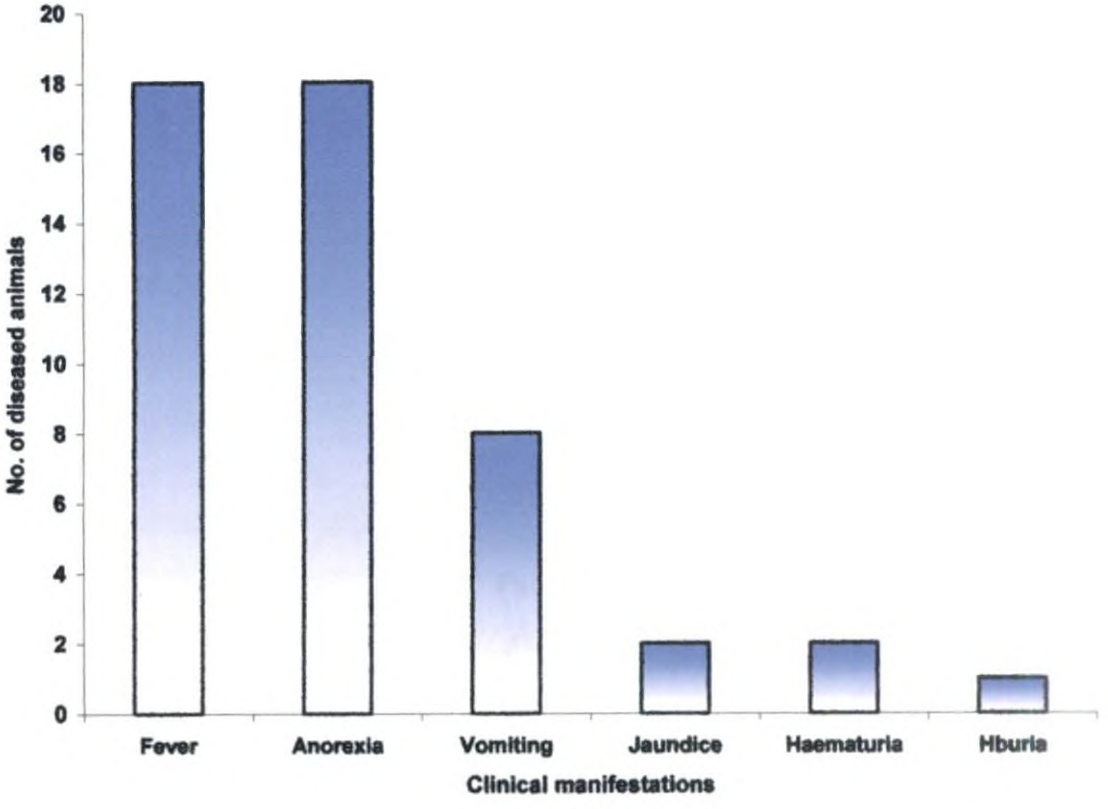

Fig. 7 Clinical manifestations of leptospirosis in dogs

4.4 CARRIER STATUS

Healthy dogs acting as a carrier of leptospires was detected in the present study. Out of the 101 healthy dogs screened for leptospirosis 18 (17.82 per cent) were found to be infected with *Leptospira*. Among the 18 healthy carriers, four (22.22 per cent) dogs were found to have leptospiremia. Renal carriage of leptospires was detected in 12 (66.67 per cent) healthy dogs, where as both leptospiremia and leptospiruria was observed in two (11.11 per cent) healthy dogs (Table 4).

4.5 DARK FIELD MICROSCOPY (DFM)

Dark field microscopic examination of blood and urine samples obtained from dogs revealed the presence of leptospires. The details of the number of samples tested and results are shown in Table (5) and Fig. (8).

Out of the 210 blood samples from dogs examined DFM detected presence of leptospires in 10 (4.76 per cent) samples. This included 3 (2.97 per cent) samples of the 101 blood samples examined from healthy dogs, where as leptospires were detected in seven (6.42 per cent) blood samples obtained from 109 diseased dogs by DFM.

Among the 210 urine samples examined DFM detected leptospires in seven (3.33 per cent) samples. Out of the 109 urine samples from diseased dogs leptospiruria was detected in the urine samples obtained from four (3.67 per cent) dogs. Of the 101 urine samples tested from healthy animals, leptospires were detected in three (2.97 per cent) samples.

4.6 SANDWICH DOT ELISA FOR LEPTOSPIRA ANTIGEN DETECTION

Sandwich dot ELISA for Leptospira antigen detection detected leptospires in the clinical samples. The number of samples tested and the results are shown in Table (5) and Fig. (9).

Among the 210 serum samples of dogs tested for pathogenic *Leptospira* organisms, 26 (12.38 per cent) were found positive. This includes 6 (5.94 per cent) out of the 101 serum samples from healthy dogs and 20 (18.35 per cent) out of the 109 serum samples from diseased dogs.

Sandwich dot ELISA for Leptospira antigen detection detected presence of pathogenic leptospires in 22 samples (10.48 per cent) out of the 210 canine urine samples tested. Of the 109 urine samples obtained from diseased dogs, eight (7.34 per cent) were found to contain pathogenic leptospires. Fourteen (13.86 per cent) urine samples out of the 101, obtained from healthy dogs were found positive for pathogenic leptospires.

4.7 SANDWICH PLATE ELISA FOR LEPTOSPIRA ANTIGEN DETECTION

The presence of leptospires in the clinical samples was detected by Sandwich Plate ELISA for *Leptospira* antigen detection. The number of samples tested and the results are given in Table (5) and Fig. (10).

Among the canine serum samples collected from 210 dogs, 26 (12.38 per cent) were found positive for *Leptospira* antigen by sandwich plate ELISA for *Leptospira* antigen detection. This included six (5.94 per cent) out of the 101 serum samples from healthy dogs and 20 (18.35 per cent) out of the 109 serum samples from diseased dogs.

Urine samples collected from 210 dogs examined by Sandwich plate ELISA for *Leptospira* antigen detection detected 22 (10.48 per cent) samples as positive for leptospires. Of which 14 (13.86 per cent) samples were from healthy dogs and eight (7.34 per cent) urine samples were of diseased dogs.

4.8 POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction (PCR) detected the presence of pathogenic leptospires in blood and urine samples collected from dogs .The details of the number tested and the results are given in Table (5) and Fig. (11).

Of the 210 serum samples collected from dogs, 26 (12.38 per cent) were found to contain the DNA of pathogenic leptospires. This included 20 (18.35 per cent) serum samples obtained from 109 diseased dogs and six (5.94 per cent) serum samples obtained from 101 healthy dogs.

Out of the 210 canine urine samples collected 22 (10.48 per cent) showed the presence of DNA of pathogenic leptospires. This includes eight (7.34 per cent) urine samples obtained from 109 diseased dogs and 14 (13.86 per cent) from healthy animals.

Table 4. Carrier status of leptospira in dogs

Source	Total No. tested	No. of dogs positive for leptospira		Leptospiremia		Leptospiruria		Both	
		No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Healthy dogs	101	18	17.82	4	22.22	12	66.67	2	11.11

Table. 5 Results of Dark field microscopy, Sandwich dot ELISA for *Leptospira* antigen detection, Sandwich plate ELISA for *Leptospira* antigen detection and Polymerase chain reaction.

Sample	No: tested	DFM		Sandwich dot ELISA		Sandwich plate ELISA		PCR	
		No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Blood/ Serum									
Healthy dogs	101	3	2.97	6	5.94	6	5.94	6	5.94
Diseased dogs	109	7	6.42	20	18.35	20	18.35	20	18.35
Total	210	10	4.76	26	12.38	26	12.38	26	12.38
Urine									
Healthy dogs	101	3	2.97	14	13.86	14	13.86	14	13.86
Diseased dogs	109	4	3.67	8	7.34	8	7.34	8	7.34
Total	210	7	3.33	22	10.48	22	10.48	22	10.48

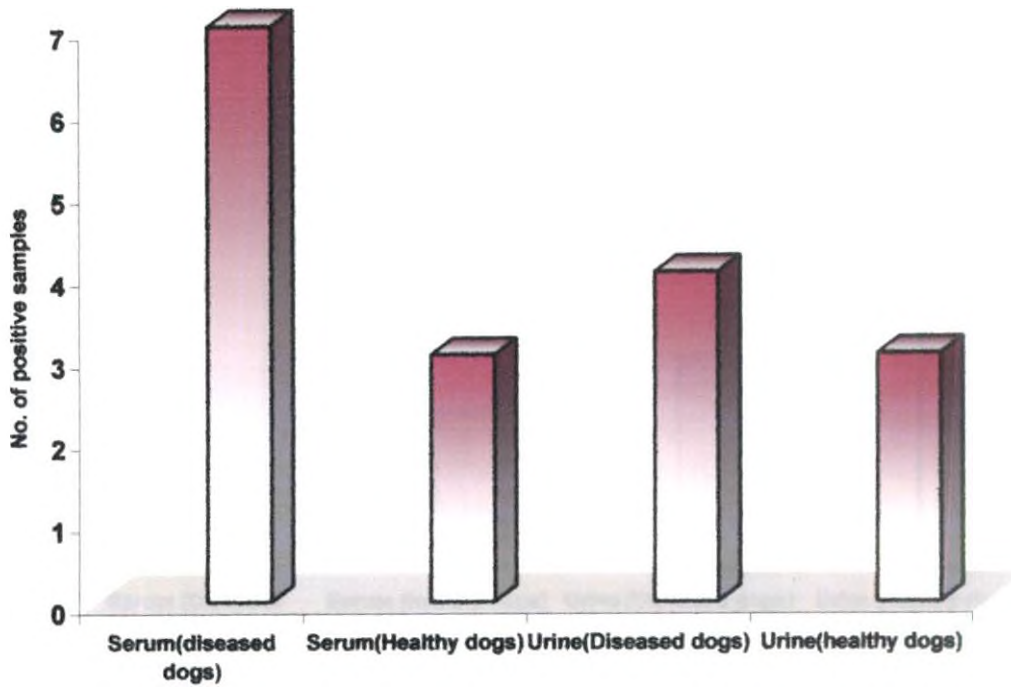

Fig. 8 Screening of dogs for leptospira using Dark field microscopy.

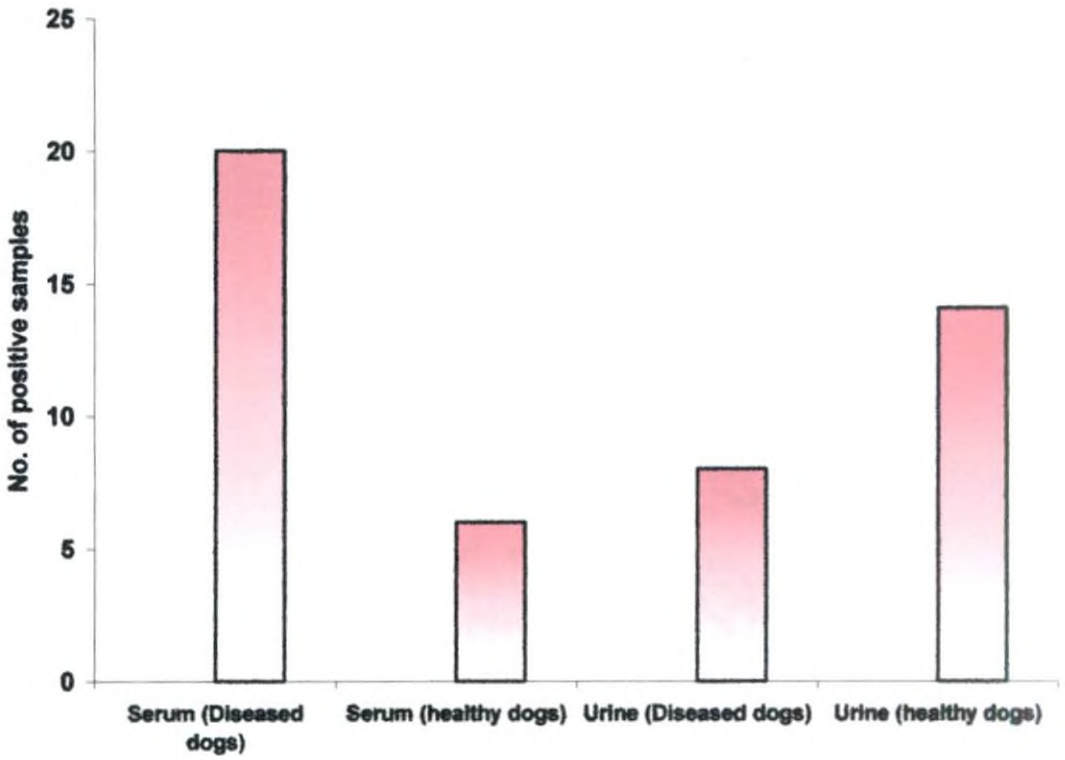

Fig. 9 Results of dogs screened for leptospira using sandwich dot ELISA for Leptospira antigen detection.

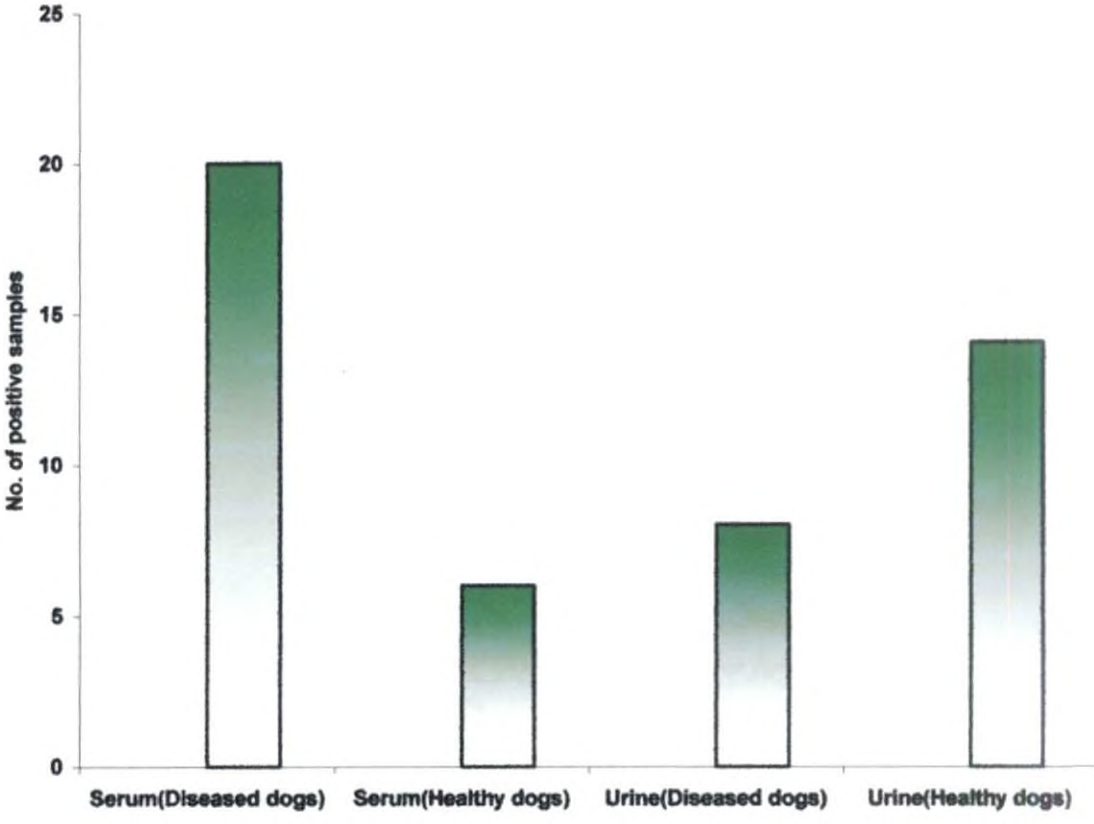

Fig. 10 Results of dogs screened for leptospira using sandwich plate ELISA for Leptospira antigen detection

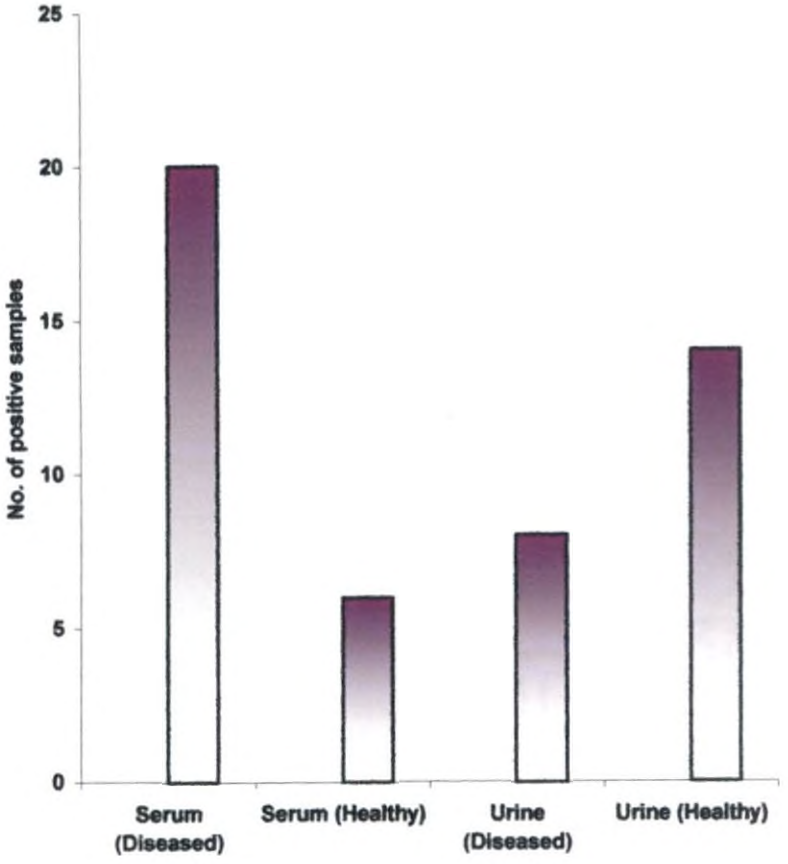

Fig. 11 Screening of dogs for *Leptospira* using Polymerase chain reaction

Discussion

5. DISCUSSION

Leptospirosis is now regarded as the most widespread anthropozoonosis in the world and the knowledge that domestic animals and pet animals can act as maintenance host for leptospires is gaining much importance. Taking into consideration of all these in the present study dogs were screened for leptospirosis to evaluate the carrier status of leptospirosis in dogs. The study also intended to assess the epidemiological factors, which favors the leptospiral maintenance in dogs.

5.1. SCREENING OF DOGS FOR LEPTOSPIROSIS

In the present study blood and urine sample were collected from 210 dogs brought to the University Veterinary hospitals at Kokkalai and Mannuthy for screening leptospirosis using darkfield microscopy, sandwich plate and dot ELISA for *Leptospira antigen detection and polymerase chain reaction.* This included 101 healthy animals and 109 diseased animals.

5.1.1 Screening of healthy dogs

Out of the 101 healthy dogs screened for leptospirosis 18 (17.82 per cent) animals were found positive. Leptospiremia alone could be detected in four (3.96 per cent) healthy animals, where as leptospiruria alone was detected in 12 (11.88 per cent) healthy dogs. Two (1.98 per cent) dogs were found to have both leptospiremia and leptospiruria.

Prevalence of leptospiral infection in apparently healthy dogs have been reported by many workers (Lieb *et al.*, 1999, Hagiwara and Rosa, 1975, Fehlert *et al.*, 2000, Chandrasekaran and Pankajalakshmi, 1997, Verma, 1982). Presence of leptospiremia in four apparently healthy dogs was detected in this study. Of which two dogs had suffered from fever, anorexia and vomiting about a month back and were treated for the same. Whereas two dogs were not having any history of previous illness suggestive of leptospirosis. Presence of leptospires in blood in

apparently healthy animals revealed by the present study is in agreement with Navarro *et al.* (1981) who found that in a group of dogs with experimentally induced leptospirosis by serovar *icterohaemorrhagiae* three dogs, from which leptospires could be isolated from blood, were not showing any apparent symptoms apart from very mild febrile reaction that occurred three days post inoculation whereas three dogs where showing only mild disease but were alert and continued to eat. Transient leptospiremia in alert and healthy dogs were reported by Tolari *et al.* (1982). He inoculated leptospiral serovar *hardjo* in three serologically negative dogs. All the three dogs developed antibody titers to MAT with a peak in 30 days after inoculation. Transient leptospiremia with no clinical signs were noted in two dogs and no leptospires could be obtained from the urine of these dogs.

The present study detected twelve healthy dogs that were excreting leptospires through urine. Out of this seven dogs were having previous illness like fever, anorexia and vomiting occurred from period ranging between two weeks to a month. Where as the rest of the lot was not having any history of illness suggestive of leptospirosis. Apparently healthy dogs, which had a previous history suggestive of leptospirosis and presently acting as leptospira carriers, have been reported by many workers (Buxton and Frazer, 1977, Greene *et al.* 1998, Noel and Kenneth, 2000). Leptospiruria in apparently healthy dogs, which were having previous history of illness suggestive of leptospirosis as revealed in this study, is in agreement with Greene *et al.* (1998), they opined that in the survivors of an acute or sub acute leptospiral infection in dogs renal colonization of leptospires will be long term, with shedding of organisms in urine for months to years. Nielsen *et al.* (1991) had found similar observations about renal carriage of leptospires in dogs. They examined two dogs with previous clinical histories suggestive of leptospirosis and found antibodies to *Leptospira interrogans* serovar *bratislava* in serum from one dog and the organism was isolated from the urine of that dog.

Leptospiruria in healthy dogs, which do not have the history of clinical signs suggestive of leptospirosis was detected and is in agreement with the findings of Hubert and Shotts (1966). They screened a kennel of 19 dogs and leptospires could

be isolated from urine of 10 dogs which do not have any history of clinical signs suggestive of leptospirosis and opined that these asymptomatic shedder dogs were harboring inapparent infections not to cause an apparent illness previous to isolation of leptospires from their urine. In a study conducted by Morter *et al.* (1959), they could detect the presence of healthy dogs as urinary shedders of leptospires, that too in dogs without any clinical history suggestive of leptospirosis. Out of the 19 dogs screened for leptospires, organism was isolated directly from the urine of one dog by cultural methods and isolations were achieved from the guinea pig inoculated with the urine from two dogs.

Healthy dogs having both leptospiremia and leptospiruria were also detected. Two healthy dogs were found to have leptospires in their blood and urine. Both of these dogs have got a previous history of illness with symptoms like fever, anorexia and vomiting. Serological evidence of concurrent infection with more than one leptospiral serovar in dogs is reported by many workers (Bishop, *et al.*1979, Marshall, 2001, Harkin *et al.* 2003a). So the possible explanation of occurrence of both leptospiruria and leptospiremia in a healthy dog is that it already had a history of illness suggestive of leptospirosis, which might have under gone a chronic course and the organisms might have localized in the renal tubules. At the same time animal might have contracted an infection of another serovar recently that is under going a sub clinical course giving us a picture of both leptospiremia and leptospiruria.

5.1.2 Screening of diseased dogs

Twenty four (22.02 per cent) out of the 109 diseased dogs were found positive for leptospirosis. This is in agreement with the findings of Venkataraman and Nedunchelliyan (1992a) who investigated an outbreak of leptospirosis in man and dog and found that out of the 94 canine sera tested 20 (21.03 per cent) were positive for leptospirosis. Venkataraman *et al.* (1991) examined sera samples of 147 dogs with the symptoms of vomiting, diarrhoea, jaundice and nephritis and found that 28 (19.04 per cent) dogs had leptospirosis.

Among the 24 diseased dogs that were found positive for leptospirosis three dogs were below one year of age, five within one to two years of age, 11 dogs two to three years old and six dogs were above three years of age. Proportionate increase in leptospirosis in dogs from aged below one year to three years as revealed by the present study is in perfect agreement with the findings of Thomas and Evans (1967) who detected similar findings while studying the distribution of leptospirosis in German shepherd dog population in the United States. Relatively low prevalence of leptospirosis in animals aged above three years is in disagreement with the findings of Ward *et al.* (2002) who detected a higher prevalence of leptospirosis in animals aged above 4 years.

Present study noticed male dogs were at significant risk of leptospirosis than the female dogs and is in agreement with the findings of Ward *et al.* (2002 and 2004).

Leptospiremia in 16 (14.68 per cent) diseased dogs were detected. This is in par with the observations made by Arimitsu *et al.* (1989), they found that, 12 dogs that were infected with leptospiral serovars *icterohaemorrhagiae* and *canicola* were showing symptoms like anorexia, depression and listlessness and out of them leptospires could be detected in the blood of only two (16.67 per cent) dogs. But the present result is not in agreement with findings of Venkataraman *et al.* (1994), they found that leptospiremia could be detected in all the five (100 per cent) clinically affected dogs that were experimentally infected with *Leptospira interrogans* serovar *icterohaemorrhagiae* during the first five days of inoculation. Adamus *et al.* (1997) reported isolation of leptospires from the blood of six (37.5 per cent) out of the 16 dogs that were suffering from chronic hepatitis associated with leptospiral infection.

Urinary shedding of leptospires was detected in four (3.67 per cent) diseased dogs screened. Urinary shedding of leptospires as found in this study is in agreement with the findings of Venkataraman and Nedunchelliyan (1992a) who observed that out of the 20 dogs found positive for leptospirosis, serovar *canicola* could be isolated from the urine of one (5 per cent) dog. In another study among 474

diseased dogs screened for leptospirosis, leptospiruria was detected in 13 (2.74 per cent) dogs that were serological reactors to leptospires (Venkataraman *et al.* 1994).

Leptospiremia and leptospiruria was found in four (3.67 per cent) diseased dogs. All these dogs were suffering from febrile illness, vomiting and depression since a week.

5.2 CLINICAL MANIFESTATIONS OF LEPTOSPIROSIS IN DOGS

The prominent clinical manifestations of canine leptospirosis revealed by this study included anorexia, fever, vomiting, jaundice and haematuria.

Fever was present in 18 (75 per cent) diseased animals, which turned out positive for leptospirosis. Buxton and Frazer (1977) opined that invasion of leptospires into host with marked leptospiremia is the initial stage in pathogenesis of leptospirosis which is characterized by a sudden onset of febrile reactions. Greene *et al.* (1998) reported that febrile reactions will be present in dogs that are undergoing an acute or a sub acute course of leptospirosis and opined that serologic or microbiologic evaluation for leptospirosis should be performed on dogs with fever of unknown origin. Fever as one of the predominant signs in dogs affected with leptospirosis as revealed by the present study is in agreement with the observations made by Verma (1982) who studied the seroepidemiology of leptospirosis in dogs in Punjab and observed that out of the 22 dogs that were positive for leptospirosis the most predominant clinical manifestation noted was fever; in 15 dogs. In contrary, fever occurring as a less predominant clinical sign of canine leptospirosis has been reported by other workers. Arimitsu *et al.* (1989) reported fever in 50 per cent of the dogs that were experimentally inoculated with leptospires. Prescott *et al.* (2002) found that only four out of the 32 dogs that were found positive for leptospirosis were having fever.

Anorexia was present in 18 (75 per cent) of diseased dogs that were positive for leptospirosis. This is in agreement with the reports of Prescott *et al.* (2002) who reported that 81 per cent of dogs that were found positive for leptospirosis had

inappetence and of Arimitsu *et al.* (1989) who found anorexia in all the 12 dogs experimentally infected with leptospiral serovars *icterohaemorrhagiae* and *canicola*.

Vomiting was present in eight (33.33 per cent) diseased dogs that were leptospira positive. Noel and Kenneth (2000) opined that vomiting and rapid dehydration could be sited as symptoms of both an acute and sub acute attack of leptospirosis in dogs. Similar observations regarding acute and sub acute leptospirosis in dogs have been mentioned by Greene *et al.* (1998). Present finding of vomiting in 33.33 per cent of diseased dogs that were found positive for leptospirosis is in disagreement with the findings of Prescott *et al.* (2002), they observed vomiting in 81 per cent of dogs that were found positive for leptospirosis.

Jaundice was noticed in two (8.33 per cent) of the diseased dogs that were found positive for leptospirosis. Inflammation of liver and pain on palpation was evident in both the cases. Greene *et al.* (1998) opined that liver is the second major parenchymatous organ damaged during leptospiremia and hepatic dysfunction occurs without major histological changes because of the subcellular damage produced by leptospiral toxins. In canine leptospirosis icteric form is a noticeable disease manifestation and is characterized by initial symptoms of pyrexia, depression and vomiting which is followed by severe jaundice that develops after three to four days and is readily noticeable on the conjunctivae, mouth, tongue and gums (Buxton and Frazer, 1977). Verma (1982) reported similar observations regarding jaundice in diseased dogs that were found positive for leptospirosis and noticed that 3 (6.38 per cent) dogs out of the 47 dogs positive for leptospirosis were suffering from jaundice. Icterus as a major clinical manifestation in dogs screened for leptospirosis was reported by Prescott *et al.* (2002), and reported occurrence of jaundice in 29 per cent of dogs screened for leptospirosis. Many workers have reported hepatic involvement in canine leptospirosis. Adamus *et al.* (1997) noticed leptospirosis with hepatic affection in beagle dogs and found that gross lesions on postmortem examinations were confined to liver that was firm and mottled and opined that there is strong connection between the presence of leptospires and the hepatic lesions. Birnbaum *et al.* (1998) observed increased liver enzyme activity in

dogs that were suffering from leptospirosis and opined that leptospiral serovars *pomona* and *grippotyphosa* are important pathogens capable of causing severe hepatic injury in dogs. Greene *et al.* (1998) found that infection of dogs with serovars *icterohaemorrhagiae* and *pomona* produce hepatic diseases and in canine leptospirosis the degree of icterus corresponds to the severity of necrotic hepatitis. Venkataraman and Nedunchelliyan (1992b) detected serovar *canicola* as the causative agent of acute fatal jaundice in a pup. Venkataraman *et al.* (1991) studied the incidence of leptospiral jaundice among dogs in Madras and found out the presence of leptospiral agglutinins in 19.04 per cent of dogs presented with jaundice and the most common serovars found were *icterohaemorrhagiae*, *canicola* and *pomona*.

Haematuria in two cases (8.33 per cent) and haemoglobinuria in one (4.17 per cent) case was detected in the diseased dogs that were screened for leptospirosis. Enlargement of the kidney and pain on palpation was evident in all these cases. Muthusethupathi (2001) attributed pathogenesis of renal failure caused by leptospirosis to three major factors namely direct bacterial invasion and direct injury caused by leptospiral endotoxin, hyaluronidase and hemolysins, non-specific factors like the combination of hypovolemia, hyperviscosity and intravascular coagulation and thirdly immunological reactions characterized by immunologlobulin deposition resulting in progressive renal failure. Similar findings of haematuria as a clinical manifestation of leptospirosis was described by Fehlert *et al.* (2000). They noticed that out of 27 beagle dogs infected with two different leptospiral serovars of serogroup *sejroe* three (11.11 per cent) dogs died after an acute clinical illness characterized by febrile temperature and haematuria. Haemoglobinuria in canine leptospirosis had been described by Thomas (1980) in a ten year old female German shepherd dog, that was infected with *Leptospira bratislava* and the dog was showing febrile reaction and abdominal pain, on exploratory laprotomy the bladder was full of dark coloured urine. A high percentage of leptospira positive cases among dogs with renal insufficiency were reported by Hagiwara and Rosa (1975), who detected 36.3 per cent reactors among dogs with renal insufficiency. Many workers reported renal involvement of varying degrees in canine leptospirosis. Sub acute nephritis in

canine leptospirosis is usually associated with the serogroups *canicola* and *icterohaemorrhagiae* (Greene *et al.*, 1998, Noel and Kenneth, 2000). Adin and Cowgill (2000) noticed that *Leptospira pomona* and *Leptospira bratislava* were the major cause of leptospirosis in dog and resulted in development of acute renal failure with mild to moderate azotemia. Harkin and Gartrell (1995) and Birnbaum *et al.*(1998) noticed acute renal failure as the most common syndrome in canine leptospirosis and the most common serovars identified for this condition were *pomona, grippotyphosa* and *autumnalis.*

The most incriminated clinical signs in canine leptospirosis are that due to the affections of liver and kidney (Green *et al.* 1998, Noel and Kenneth, 2000). Nonspecific clinical signs like anorexia and vomiting as predominant clinical signs of canine leptospirosis as revealed by this study is in agreement with the findings of Prescott *et al.* (2002) as they noticed lethargy, inappetence and vomiting as the predominant clinical signs of leptospirosis in dogs. Rentko and Ross (1992) also observed similar findings in canine leptospirosis and opined that this may be due to infection with different serovars than those previously reported.

5.3 VACCINATION STATUS IN DOGS SCREENED FOR LEPTOSPIROSIS

Out of the total 42 dogs found positive for leptospirosis, proper vaccination status was present only for 15 (35.71 per cent) animals. This included seven (16.67 per cent) healthy and eight (19.05 per cent) diseased dogs. Present finding of leptospiral infection even in vaccinated dogs is in agreement with the findings of Greene *et al.* (1998) and Noel and Kenneth (2000). They opined that the commonly used bivalent bacterins that contain two main serovars *canicola* and *icterohaemorrhagiae* did not provide protection against other disease causing serovars such as *grippotyphosa, pomona, hardjo* and *bratislava.* Indu (1997) detected serovar *pomona* as the most prominent serovar in Thrissur area and serovars *canicola* and *icterohaemorrhagiae* were found as the less incriminated ones. So the prevalence of the serovar not included in the currently used leptospiral vaccine gives satisfactory explanation for leptospirosis even in properly vaccinated

dogs. The presence of leptospiral infection in apparently healthy dogs that were properly vaccinated is in perfect agreement with observations of Greene *et al.* (1998), they stated that immunization had been effective in reducing the severity and prevalence of canine leptospirosis but it will not prevent the carrier status that is associated with potential zoonotic risk.

Eight (19.05 per cent) animals, two (4.76 per cent) healthy and six (14.29 per cent) diseased were having an improper vaccination status, with vaccines taken in their early life with delay in the annual booster vaccinations. Earlier report of Thomas (1980) is in agreement with the present findings of leptospirosis in dogs with an improper vaccination status. He noticed *Leptospira bratislava* infection in a ten year old dog that was having a history of vaccination against leptospirosis in its 12^{th} and 14^{th} weeks of age. Noel and Kenneth (2000) also suggested yearly booster vaccination against leptospirosis for at risk dogs and immunity may not even last a complete year.

Fifteen (35.71 per cent) of the leptospira positive animals, which included nine (21.42 per cent) healthy and six (14.29 per cent) diseased dogs, were not at all vaccinated. No history regarding vaccination status was available with four (9.52 per cent) diseased dogs presented.

5.4 CARRIER STATUS

Healthy dogs acting as a carrier of leptospires was detected. Out of the 101 healthy dogs screened for leptospirosis 18 (17.82 per cent) were found to be infected with *Leptospira.* Similar work done by Harkin *et al* (2003b) found that irrespective of health status 8.2 per cent of dogs were affected with leptospirosis.

Four (22.22 per cent) dogs were having leptospiremia that were showing no apparent clinical signs. Similar findings of leptospiremia in apparently healthy dogs have been reported by Navarro *et al.* (1981) who found that in a group of dogs with experimentally induced leptospirosis by serovar *icterohaemorrhagiae* three dogs, were having leptospiremia without showing any apparent symptoms apart from very

mild febrile reaction where as another three dogs where showing only mild disease but were alert and continued to eat. Transient leptospiremia with no clinical signs as detected in the present study is in agreement with the findings of Tolari *et al.* (1982) who detected the same in two out of the three dogs experimentally infected with leptospires.

Renal carriage of leptospires in healthy dogs with shedding of leptospires in their urine was detected in 12 (66.67 per cent) dogs screened. This included dogs that were having previous clinical history suggestive of leptospirosis and those with out such history. Many workers have reported similar findings. Hubert and Shotts (1966) detected renal carriage of leptospirosis in ten healthy dogs in a kennel that were not having any history of illness suggestive of leptospirosis. Similar observations were made by Morter *et al.* (1959) and they detected three urinary shedders of leptospires among 19 healthy dogs.

Dogs with previous clinical history of leptospirosis, acting as renal carriers as found in this study is in agreement with the findings of Greene *et al.* (1998) who opined that in survivors of leptospirosis in dogs, organisms can localize in the kidney resulting in long term shedding of the organisms in urine. Similar observations were made Nielsen *et al.* (1991) who could isolate leptospires from the urine of a healthy dog that had a clinical history suggestive of leptospirosis.

Present study revealed healthy carrier dogs with both leptospiremia and leptospiruria as observed in two (11.11 per cent) healthy dogs. The observations made by Navarro *et al.* (1981) and Tolari *et al.* (1982) regarding healthy dogs having transient leptospiremia and those made by Morter *et al.* (1959) and Hubert and Shotts (1966) regarding renal carriage in healthy dogs gives sufficient explanation for the present finding of leptospiremia and leptospiruria in healthy carrier dogs.

5.5 DARK FIELD MICROSCOPY (DFM)

Among the 210 blood samples tested by DFM, leptospires could be detected in 10 (4.76 per cent) blood samples. This included three (2.97 per cent) samples from healthy dogs and seven (6.42 per cent) samples obtained from diseased dogs. All the diseased animals in which DFM detected leptospiremia were in the first week of their illness. This is in agreement with Cruickshank *et al.* (1975) who opined that during the first week of infection leptospires may be detected by dark ground microscopic examination of untreated blood. Detection of leptospires in the blood from a dog that was suffering from fever was reported by Chandrasekaran and Pankajalakshmi (1997). Leptospiremia in three (2.97 per cent) symptomless healthy dogs as seen in the present study were in agreement with the findings of Chandrasekaran and Pankajalakshmi (1997), who detected leptospiremia in 1 (33.33 per cent) healthy dog out of the three dogs tested.

Among the urine samples examined DFM detected leptospires in seven (3.33 per cent) samples. This included four (3.67 per cent) samples from diseased dogs and three (2.97 per cent) samples tested from healthy animals (Table 5, Fig. 3). Detection of leptospires in the urine of 3.67 per cent of urine samples from diseased dogs in the present study is in agreement with the findings of Venkataraman *et al.* (1994) who detected presence of leptospires in 2.74 per cent of the urine samples tested from diseased dogs. In the present study DFM detected leptospires in the urine of three (2.97 per cent) healthy dogs (Table 5, Fig. 3).

5.6 SANDWICH DOT ELISA FOR Leptospira ANTIGEN DETECTION

Out of the 210 serum samples of dogs tested for pathogenic *Leptospira* organisms, 26 (12.38 per cent) were found positive. This included 6 (5.94 per cent) out of the 101 serum samples from healthy dogs and 20 (18.35 per cent) out of the 109 serum samples from diseased dogs.

Sandwich dot ELISA for *Leptospira* antigen detection detected presence of pathogenic leptospires in 22 samples (10.48 per cent) out of the 210 canine urine samples tested. Of the 109 urine samples obtained from diseased dogs, eight (7.34 per cent) were found to contain pathogenic leptospires. Fourteen (13.86 per cent) urine samples out of the 101, obtained from healthy dogs were found positive for pathogenic leptospires. Sureshbabu *et al.* (1996) reported the standardization of a double antibody sandwich dipstick ELISA for leptospiral antigen detection and opined that so far, dipstick ELISA was not available for leptospiral antigen detection. They detected leptospires in eight (26.6 per cent) samples out of the 30 human urine samples and on the corresponding serum samples a positive titer of leptospiral agglutinins were observed, but dip stick ELISA failed to detect any leptospires in urine samples collected from seven other cases, whose sera gave positive MAT titers. Monoclonal antibody based leptospiral antigen detection in urine of human beings was reported by Saengjaruk *et al.* (1996). They used Monoclonal antibody LD5 in a dot ELISA for leptospira antigen detection in urine samples collected from human beings.

5.7 SANDWICH PLATE ELISA FOR LEPTOSPIRA ANTIGEN DETECTION

Among the canine serum samples collected from 210 dogs, 26 (12.38 per cent) were found positive for *Leptospira* antigen by sandwich plate ELISA for *Leptospira* antigen detection. This included six (5.94 per cent) out of the 101 serum samples from healthy dogs and 20 (18.35 per cent) out of the 109 serum samples from diseased dogs.

Urine samples collected from 210 dogs examined by Sandwich plate ELISA for *Leptospira* antigen detection detected 22 (10.48 per cent) samples as positive for leptospires. Of which 14 (13.86 per cent) samples were from healthy dogs and eight (7.34 per cent) urine samples were of diseased dogs.

5.8 POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction (PCR) detected the presence of pathogenic leptospires in blood and urine samples collected from dogs. PCR was successfully used for rapid diagnosis of leptospirosis, concerned with detection of leptospiral DNA in body fluids like aqueous humor (Merien *et al.*, 1992) cerebrospinal fluid (Romero *et al.*, 1998) and in semen (Heinemann *et al.*, 1999).

Of the 210 serum samples collected from dogs, 26 (12.38 per cent) were found to contain the DNA of pathogenic leptospires. This included 20 (18.35 per cent) serum samples obtained from 109 diseased dogs and 6 (5.94 per cent) serum samples obtained from 101 healthy dogs (Table 5, Fig. 6). The present findings is in disagreement with the findings of Senthilkumar *et al.* (2001) who detected leptospires in 43.47 per cent blood samples collected from dogs.

Out of the 210 canine urine samples collected 22 (10.48 per cent) showed the presence of DNA of pathogenic leptospires. Senthilkumar *et al.* (2001) detected leptospires in 50.0 per cent urine samples collected from dogs. The present study revealed the presence of leptospiral DNA in eight (7.34 per cent) urine samples obtained from 109 diseased dogs. The present finding is in agreement with Harkin *et al.* (2003b) who detected leptospiral DNA in eight (6.06 per cent) out of the 132 canine urine samples, from dogs with clinical signs suggestive of leptospirosis.

Among the 101 urine samples obtained from healthy dogs, 14 (13.86 per cent) were positive for leptospires using polymerase chain reaction. In a similar study conducted by Harkin *et al* (2003a) found 37 (7.04 per cent) healthy dogs excreting leptospires in their urine out of the 500 dogs screened using polymerase chain reaction.

5.9 COMPARISON OF DARK FIELD MICROSCOPY, SANDWICH DOT ELISA FOR LEPTOSPIRA ANTIGEN DETECTION, SANDWICH PLATE ELISA FOR LEPTOSPIRA ANTIGEN DETECTION AND POLYMERASE CHAIN REACTION.

In the present study a total of 210 blood and urine samples obtained from both healthy and diseased dogs were examined for leptospires using Dark field microscopy, Sandwich dot and plate ELISA for *Leptospira* antigen detection and Polymerase chain reaction.

Out of the 210 blood samples tested, ten (4.76 per cent) samples were found positive by DFM. Among the serum samples 26 (12.38 per cent) samples were found positive by Sandwich dot and plate ELISA for *Leptospira* antigen detection and Polymerase chain reaction. Among the 210 urine samples tested, seven (3.33 per cent) samples found positive by DFM, where as 22 (10.48 per cent) urine samples were found positive by Sandwich dot and plate ELISA for *Leptospira* antigen detection and Polymerase chain reaction.

The comparison of the results of DFM, Sandwich dot and plate ELISA for *Leptospira* antigen detection and Polymerase chain reaction revealed that, the urine samples found positive by DFM was found positive on the other tests also. In addition these tests could detect presence of leptospiral antigen in urine samples from another 15 dogs in which DFM failed to detect any leptospires in the urine.

Sandwich dot and plate ELISA for *Leptospira* antigen detection and Polymerase chain reaction could detect leptospiral DNA in 26 (12.38 per cent) serum samples where as DFM detected leptospires in only 10 (4.76 per cent) samples, indicating that the other two tests detected presence of leptospires in 16 samples on which DFM failed to detect any leptospires.

The relative inferiority of DFM as a diagnostic tool of leptospirosis have been described by many workers. Sehgal (2001) in a study observed that DFM showing sensitivity and specificity of approximately 40 per cent in positive cases

and hence it should not be used as a sole diagnostic technique in the diagnosis of leptospirosis. The failure of DFM to detect active leptospiral infections if the number of organisms are less than 10^5 /ml and the intermittent shedding of leptospires in the urine of carrier animals as opined by Greene *et al.* (1998) may be the reasons for an inferior result for DFM in the present study.

Comparison of the results of Sandwich dot and plate ELISA for leptospira antigen detection and polymerase chain reaction revealed that there is hundred per cent agreement between these tests. All the urine and blood samples that gave positive for leptospira antigen on dot ELISA were positive on plate ELISA also. This was in perfect agreement with the findings of samples examined by PCR. The relative superiority of polymerase chain reaction in diagnosis of leptospirosis over conventional techniques were reported by many workers (Harkin *et al.* 2003a, Van Eys *et al.* 1989, Merien *et al.* 1992). So far no literature is available regarding a comparative study between PCR and antigen detection ELISA for leptospirosis. The equivocal results obtained between PCR and antigen detection ELISA used in the present study clearly indicated that it could be used as an effective tool in the diagnosis of leptospirosis.

To conclude, the present study revealed 42 dogs as positive for leptospirosis out of the 210 dogs screened. Eighteen healthy dogs acting as carriers of leptospirosis were detected in the present study. The predominant clinical manifestation of canine leptospirosis revealed in the present study was nonspecific signs like fever, anorexia and vomiting suggesting that dogs with such non specific signs should also be suspected for leptospirosis. Present study revealed that male dogs and dogs' aged from six months to two and a half years are at increased risk of leptospirosis. Comparative evaluation of the diagnostic tests namely DFM, sandwich plate and dot ELISA and PCR for leptospira diagnosis in dogs revealed the inferior nature of DFM as a diagnostic tool for leptospirosis and the new, cost effective and easy to perform antigen detection ELISA were showing perfect agreement with PCR in leptospira diagnosis. So on the light of present findings the antigen detection

ELISA used in this study can be adopted for leptospira diagnosis even in the field level where sufficient laboratory infrastructure is lacking.

Summary

6. SUMMARY

Leptospirosis is an acute anthropozoonotic infection of worldwide significance caused by spirochaete *Leptospira interrogans* which has 23 serogroups and >200 serovars. Various factors influencing the animal activity, suitability of the environment for the survival of the organism and behavioral and occupational habits of human beings can be the determinants of incidence and prevalence of the disease. The disease was considered inconsequential till recently, but it is emerging as an important public health problem during the last decade or so due to sudden upsurge in the number of reported cases and outbreaks.

The present study was envisaged to assess the carrier status in dogs for leptospirosis, to compare the efficacy of dark field microscopy, Sandwich dot ELISA for leptospira antigen detection, Sandwich plate ELISA for leptospira antigen detection, and Polymerase chain reaction in detecting leptospires in dogs and to evaluate the epidemiological factors that favor the maintenance of leptospires in the canine population. Serum and urine samples collected from 210 animals both healthy and diseased with any of the symptoms suggestive of leptospirosis like fever, anorexia, vomiting, jaundice, haematuria and haematemesis, brought to the University Veterinary hospitals at Mannuthy and Kokkalai formed the materials of study. The epidemiological data regarding sex, age, vaccination status against leptospirosis, history of any previous illness suggestive of leptospirosis and the present clinical status were collected.

Out of the 210 dogs screened 101 were healthy dogs and 109 were diseased dogs. The results of the present investigation showed that 42 dogs out of the 210 dogs screened were positive for leptospirosis. This included 18 healthy dogs and 24 diseased dogs. Leptospiremia alone could be detected in 16 (14.68%) diseased dogs, and in four (3.96%) healthy dogs. Leptospiruria alone was detected in 12 (11.88%) healthy dogs and in four (3.67%) diseased dogs presented. Presence of both

leptospiremia and leptospiruria was detected in four (3.67%) diseased and two (1.98%) healthy dogs.

The present study revealed fever, anorexia, vomiting, jaundice, haematuria and haemoglobinuria as the predominant symptoms of leptospirosis in dogs. Fever and anorexia was present in 18 (75%) animals where as vomiting was present in eight (33.33%), jaundice in two (8.33%), haematuria in two cases (8.33%) and haemoglobinuria was present in one (4.17%) case.

Present study also noticed male dogs at a greater risk of leptospirosis than the female dogs. A proportionate increase in the prevalence of leptospirosis was noted among dogs aged six months to two years and relatively lower prevalence was noted in dogs above three years of age.

Enquiry of the vaccination status against leptospirosis of the dogs, which were positive for leptospirosis, revealed that leptospirosis is prevalent in equal proportion (15 numbers each) in the vaccinated and nonvaccinated dogs. Eight dogs, which were found positive for leptospirosis, were having an improper vaccination history with vaccines taken during early stages of their life and no history regarding vaccination was available with four dogs presented.

The present study detected healthy dogs acting as carriers of leptospira. Out of the 109 dogs screened for leptospirosis 18 were found positive for leptospirosis. Fourteen dogs among them were having renal carriage of leptospires, excreting the leptospires through urine. Where as four animals were showing the presence of leptospires in blood. Both leptospiremia and leptospiruria was detected in two healthy dogs.

Among the four diagnostic tests used in the present study for screening dogs, dark field microscopy turned out to be inferior in finding out leptospires in clinical samples from both healthy and diseased dogs. Out of the total 210 blood samples tested, DFM detected leptospires in only 10 (4.76%) samples, three (2.97%) from healthy and seven (6.42%) obtained from diseased dogs. Out of the 210 urine

samples screened DFM detected leptospires in seven (3.33%) samples, four (3.67%) from diseased and three (2.97%) from healthy dogs. Whereas the sandwich dot and plate ELISA for Leptospira antigen detection and the polymerase chain reaction, which were showing hundred percentage agreements in their results, were proven to be superior techniques in finding out leptospiral infection in dogs. These tests revealed the presence of leptospires in 26 (12.38%) out of the 210 serum samples screened which included 6 (5.94%) out of the 101 serum samples from healthy dogs and 20 (18.35%) out of the 109 serum samples from diseased dogs. Among the 210 urine samples screened, presence of leptospires were detected in 22 samples (10.48%) that included eight (7.34%) among 109 urine samples obtained from diseased dogs and fourteen (13.86%) urine samples out of the 101, obtained from healthy dogs.

Hence the present study revealed that the newly devised, easy to perform and cost effective sandwich dot and plate ELISA for leptospira antigen detection can be adopted as effective diagnostic tools for canine leptospirosis even at the field level where sophisticated infrastructure is lacking.

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SCREENING OF DOGS FOR LEPTOSPIRA USING SANDWICH DOT ELISA

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ABSTRACT

An investigation was carried out in 210 dogs of Thrissur district to assess the prevalence of leptospirosis using diagnostic techniques like darkfield microscopy, Sandwich dot ELISA for Leptospira antigen detection, Sandwich plate ELISA for Leptospira antigen detection and Polymerase chain reaction. Sera and urine samples were collected from dogs brought to University Veterinary Hospitals, Kokkalai and Mannuthy that included 109 diseased animals and 101 healthy dogs. Out of the 210 sera samples tested 26 (12.38 per cent) samples, six (5.94 per cent) out of the 101 samples from healthy dogs and 20 (18.35 per cent) out of the 109 serum samples from diseased dogs were found positive. Out of the 210 urine samples tested 22 samples (10.48 per cent), eight (7.34 per cent) from diseased dogs and 14 (13.86 per cent) from healthy dogs were found positive for pathogenic leptospires. Prevalence of leptospirosis was found higher in male dogs and in dogs aged from six months to three years. The present finding of healthy carriers for leptospirosis among dogs shows the need of checking dogs more carefully for inapparent leptospirosis because of the human health hazard. Evaluation of the comparative efficacy of the four diagnostic tests revealed darkfield microscopy inferior in detecting leptospires in dogs and the newly developed, easy to perform and cost effective sandwich dot and plate ELISA for Leptospira antigen detection that was showing hundred percent agreements with polymerase chain reaction can be adopted as an effective diagnostic tool for canine leptospirosis even at field level where sophisticated infrastructure is lacking.

Appendix

Department of Veterinary Epidemiology and Preventive Medicine

College of Veterinary and Animal Sciences, Mannuthy.

Proforma – 1

Date .....

University Veterinary Hospital – Mannuthy/Kokkalai

Name and Address of Owner :

Phone No. :

Details of the animal

Species: Breed: Age: Sex:

Vaccination status (against Leptospirosis):

1st :

2nd :

3rd :

4th and subsequent :

Is there any delay in vaccination : Y / N

Vaccination status against any other disease :

Any history of previous illness/Leptospirosis

With symptoms like

a) Fever : Y / N
b) Anorexia : Y / N
c) Vomiting : Y / N
d) Haematuria : Y / N
e) Jaundice : Y / N
f) Haematemesis : Y / N

Present complaint of the owner

a. Fever : Y / N
b. Anorexia : Y / N
c. Vomiting : Y / N
d. Haematuria : Y / N
e. Jaundice : Y / N
f. Haematemesis : Y / N

Observations

1. Jaundice :
2. Pulse :
3. Palpation of kidney/Liver : Normal /Enlarged /Painful /Contracted

Clinical samples collected :

Results of laboratory examinations :

1. D.F.M :
2. Sandwich dot ELISA :
3. Sandwich plate ELISA :
4. Polymerase chain reaction :

Signature