CLINICO-THERAPEUTIC STUDIES ON LEPTOSPIROSIS IN DOGS

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DECLARATION

I hereby declare that the thesis entitled **"CLINICO-THERAPEUTIC STUDIES ON LEPTOSPIROSIS IN DOGS"** is a bonafide record of research work done by me during the course of research and that this 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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3-5-2010

CERTIFICATE

Certified that this thesis, entitled **"CLINICO-THERAPEUTIC STUDIES ON LEPTOSPIROSIS IN DOGS"** is a record of research work done independently by **Riyas M.A.,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, associateship or fellowship to him.

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EXTERNAL EXAMINER

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Riyas M.A.

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Introduction

1. INTRODUCTION

Leptospirosis is a zoonosis of world wide distribution caused by infection with pathogenic spirochetes of genus *Leptospira*. Infections are prevalent in wild and domestic animals.

Historically, the genus *Leptospira* has been divided into more than 300 serovars belonging to two species: *Leptospira interrogans* sensu lato and *Leptospira biflexa* sensu lato, containing pathogenic and saprophytic strains, respectively. Serogroups comprise antigenically related serovars and 24 serogroups have been described for pathogenic strains. Currently a classification based on DNA identification is in use and based on genetic homology in DNA hybridization experiments, at least 16 genomic species have been described in the genus *Leptospira* and resulting in a family comprising 13 pathogenic *Leptospira* species with more than 260 serovars and six saprophytic *Leptospira* species contain more than 60 serovars.

These serovars are maintained in nature by numerous sub clinically infected wild and domestic animal reservoir hosts that serve as a potential source of infection and illness for people and other incidental animal hosts. Leptospirosis has been recognized as a re-emerging infectious disease, in part because of recent large scale outbreaks associated with recreational activities. A primary reservoir host exists for each serovar which maintains the organism's survival and dissemination in the environment. The recognized primary reservoir hosts for serovars infecting include dog (canicola) rat (icterohaemorrhagiae), cow (hardjo) and swine and cow (pomona) although all mammals are susceptible to infection by any serovar, clinical signs are most severe with non host adapted serovars. The most commonly incriminated serovars in infection with canine leptospirosis have been canicola, icterohaemorrhagiae, grippotyphosa, pomona, and bratislava, which belong to serogroups canicola, icterohaemorrhagiae, grippotyphosa, pomona and australis, respectively.

Disease incidence or outbreaks often increase during periods of higher rainfall or flooding, especially in tropical regions. In arid areas or during drought conditions, infections of accidental hosts are more common around water sources. Primary route of infection are direct contact (oral or conjunctival), venereal and placental transfer, bite wounds, and ingestion of infected or contaminated meats.

Four syndromes have been identified in dogs: icteric, haemorrhagic, uraemic (Stuttgart disease) and reproductive (abortion and birth of premature or weak pups). Generally, infections of dogs with serovars canicola, bratislava and grippotyphosa have been associated with predominantly renal dysfunction and with minimal liver involvement, whereas serovars icterohaemorrhagiae and pomona produce more hepatic disease. Younger dogs (under 6 months) seen to develop more signs of hepatic dysfunction in any disease outbreak. In dogs, infection with *Leptospira interrogans* serovar Canicola or *L. interrogans* serovar Icterohaemorrhagiae typically causes a hepatonephric syndrome that is characterized by acute haemorrhagic diathesis, sub acute icterus, or sub acute uraemia.

The Microscopic Agglutination Test (MAT) is considered to be the standard serological test used for diagnosing leptospirosis. Dark field microscopy can detect live leptospires in a wet mount of fresh urine. A minimum of 10^5 organisms/ml must be present. Despite its widespread usage and international recognition, MAT is not commonly employed for diagnosis since it can only be performed in a few referral laboratories and requires analysis of paired sera to achieve sufficient sensitivity. Sensitivity of polymerase chain reaction (PCR) often precludes the need for isolation and culture, thus making it ideal for the detection of organisms involved in acute infections. Direct fluorescent antibody (FA) techniques have been adapted to identify leptospiral serovars in tissue imprints of liver and kidney and in body fluids such as blood or urine.

During canine leptospiraemia, antibiotics of choice are penicillin G, ampicillin, or amoxicillin, with dosages adjusted for degree of renal insufficiency. Doxycyline is often used in dogs especially during leptospiruria. Vaccination protects the animal against acute signs but may not prevent infection, if the dog is exposed to a high infectious challenge or to a highly invasive strain.

Inactivated vaccines used for dog immunization contain *L. canicola* and *L. icterohaemorrhagiae* bacterins because leptospirosis in dog has been historically associated with these serovars. Use of vaccines containing *L. interrogans* serovars Canicola and Icterohaemorrhagiae has markedly reduced the incidence of disease attributable to them.

Widespread use of a bivalent vaccine against serovars canicola and icterohaemorrhagiae decreased the incidence of homologous infections with both serovars, but infections with other serovars have been reported to cause acute clinical infections in dogs in Europe and the USA. Survival rates for dogs with leptospirosis range from 78-88 per cent.

Taking into consideration with above all factors and its difficulty in diagnosis, the present study deals with

1. Assessment of the occurrence of leptospirosis in dogs.

2. Study the clinico-haematological and biochemical changes in leptospirosis.

3. Evaluation of the efficacy of treatment with Amoxicillin – Clavulanic acid and Benzyl penicillin.

Review of Literature

2. REVIEW OF LITERATURE

2.1 History

Weil (1886) described a clinical syndrome characterized by splenomegaly, jaundice, haemorrhages and nephritis. This syndrome is usually referred to as Weil's disease and this has become synonymous with leptospirosis. Leptospirosis had various names in different parts of the world that denoted seasonal association, symptoms, duration or occupations that were thought to be associated with the disease.

Inada and Ido successfully transmitted the infection to guinea pigs and from the blood of the infected animals they grew the responsible organism. The Japanese workers who discovered the organism responsible for Weil's disease named it *Spirochaeta icterohaemorrhagiae*. Naguchi introduced the genus *Leptospira* on account of the difference in morphology and movement. He described the characteristic feature of this organism as 'long, slender, cylindrical, highly flexible filament with tightly set, regular, shallow spirals'. The family Leptospiraceae among the Order of Spirocheatales was proposed by Pillot and Ryter (Sehgal, 2007).

By 1940s leptospirosis in animals was recognized as an important veterinary problem as well as a source of infection to man. It has now proved that almost any mammalian species including wild animals and aquatic mammals can harbour leptospires and can act as source of infection to man. The studies showed that leptospires adhere to platelets and this adhesion was thought to be the cause of thrombocytopenia and haemorrhages seen in Weil's disease.

Before the discovery of antibiotics, specific therapy was limited to immunotherapy using rabbit or horse antisera and arsenicals. Since rats were the first recognized animal carrier of leptospires, the initial attempts at control of the disease were centred on rodent control. Once it was understood that a wide

variety of animals can harbour the organism and act as the source of infection to man, the difficulties in control became obvious. In India, reports of Weil's disease had started appearing in the literature by the end of 19th century. The first report of bacteriologically confirmed cases of leptospirosis originated from the Andaman Islands (Taylor and Goyle, 1931).

2.2 Occurrence of leptospirosis in dogs

2.2.1 International

Ward (2002) based on a retrospective study to describe seasonal patterns of leptospirosis in the United States and Canada, observed the disease had a seasonal distribution (late summer to fall), and rainfall could be used to predict the occurrence of leptospirosis.

Boqvist *et al*. (2003) based on the indirect fluorescent antibody test, pathological and serological findings reported that *Leptospira* infection was common among fattening pigs in the Mekong delta in southern Vietnam**.**

Lilenbaum *et al.* (2008) screened for leptospirosis in thirteen goat herds and seven sheep flocks in the state of Rio de Janeiro, Brazil and reported the seroprevalence of 20.9 per cent and 13.7 per cent for goats and sheep, respectively, and the predominance of hardjo serovar, were noticed in this population.

Serum antibody titers against the prevailing serovars of *Leptospira interrogans* were evaluated in 52 dogs by microscopic agglutination test in the South western Nigeria and found that new non vaccinal serovars of grippotyphosa, pomona and bratislava were of higher in prevalence than the old vaccinal serovars of canicola and icterohaemorrhagiae. This could be due to growing pet-wildlife contact in urbanization that exposes the pets to infection with these former animal serovars (Okewole and Ayoola, 2009).

2.2.2 National

Govindarajan *et al.* (2007) reported seroprevalence of leptospirosis in 65 per cent canine sample tested in Madras Veterinary College, Chennai.

Velineni *et al.* (2007) in a retrospective study in human beings with leptospirosis in Hyderabad and Andhra Pradhesh, reported the predominant serogroup was icterohaemorrhagiae (68 per cent).

Balakrishnan *et al*. (2008) reported leptospirosis in 61.54 per cent of dogs in Tamilnadu.

Investigations conducted by *Leptospira* laboratory of IVRI had revealed that since 1995 the seroprevalence of leptospirosis in various states had been 5.4 per cent in buffaloes, 7.5 per cent in cattle, 12.5 per cent in sheep, 14.6 per cent in horses and 15.9 per cent in dogs (Srivastava, 2008).

2.2.3 State

Indu (1997) reported a seropositivity of 32.66 per cent where as Nair (2004) reported 12.38 per cent for leptospires among dogs in and around Thrissur district.

A seroprevalence of 36.36 per cent leptospiral antibodies among dogs and 47 percent leptospiral antibodies in cattle found in a study in Thrissur district, Kerala (Soman, 2004).

Swapna *et al*. (2006) in a cross sectional study reported a seroprevalence of 37 per cent leptospiral antibodies in high risk group of patients at Calicut, North Kerala.

Seroepidemiological study of leptospirosis in the two hamlets of Ernakulam district in Kerala detected leptospiral antibodies in 29.4 per cent persons and 11 per cent of rodents in the two areas surveyed (Kuriakose *et al*., 2008).

2.3 Epidemiology

In all surveys of domestic animals, antibody prevalence in sera was much more common than clinical illness, suggesting that sub clinical infections occur (Songer and Thiermann, 1988).

Indu (1997) reported 53.06 per cent *Leptospira pomona* and 32.65 per cent *Leptospira canicola* antibodies in MAT positive sera samples among dogs in and around Thrissur district, Kerala.

Results of several studies indicated that serovars pomona, grippotyphosa, and bratislava were presently prevalent and responsible for most cases of canine leptospirosis (Birnbaum *et al*., 1998).

Adin and Cowgill (2000) in a retrospective study found that affected dogs were predominantly large breed males and more incidences noted on Labrador Retrievers and German Shepherd dogs. Majority of the affected dogs lived in an urban or suburban environment.

Boutilier *et al*. (2003) in a retrospective study demonstrated that the predominant serovar in clinical cases of leptospirosis was *L. grippotyphosa* (72.1 per cent). The dogs in this series were 3 to 10 years of age with a mean of 5.5 years. The gender distribution included three spayed females, four intact females, three castrated males and five intact males and there was no apparent breed preference.

Soman (2004) reported 38.63 per cent sera samples of dogs were positive for *Leptospira pomona* and 36.36 per cent for *Leptospira australis* among the total positive sera samples collected from Thrissur district, Kerala.

Middle aged dogs may be more active outside their normal home environment than young or old dogs, increasing potential exposure to leptospira serovars (Ward *et al*., 2004).

In a retrospective study in 42 dogs with leptospirosis identified predominant serogroup as grippotyphosa (31 per cent). The mean age of affected dogs was 4-8 years (0.2 to 14 years). The most commonly represented breeds were Bernese mountain dogs and Dachshunds. 60 per cent were male and 40 per cent were female dogs (Geisen *et al*., 2007).

Miller *et al*. (2007) in a retrospective study of leptospirosis in affected dogs found an increase in case fatality rate in males compared with females and also in animals with jaundice compared with those animals without jaundice.

In a retrospective study of seven clinical cases of dogs diagnosed with leptospirosis the highest antibody titres were found against *Leptospira* serogroup pomona and javanica. Among affected cases five were intact males, two were intact bitches. They ranged in age from four months to nine years (Claus *et al.,* 2008).

Kerala gets heavy rains and intermittent floods from April to October. Intermittent flooding of low lying areas leads to repeated flushing out of the forests and farmlands and the rodent burrows there into all water sources including ponds, streams, rivers and canals where leptospires can survive for months. This contamination of surface water can cause monsoon outbreaks as well as sporadic cases throughout the year (Kuriakose *et al*., 2008).

Okewole and Ayoola (2009) reported the prevalence of grippotyphosa was singularly higher in the large adult male dogs among the local and exotic breeds tested by microscopic agglutination test and observed over representation of the German Shepherd breed (42.6 per cent) of dogs.

2.4 Transmission

Leptospiral organisms are shed in the urine of infected animals and can survive for long periods in surface water. Susceptible hosts are infected through

contact with contaminated water. Bacteria enter through the damaged skin or mucous membranes of exposed animals (Adin and Cowgill, 2000).

Animals recovering from leptospirosis may become asymptomatic carriers harbouring virulent leptospires in the renal tubules for extended periods and shedding infectious leptospires into the environment (Levett, 2001).

Dogs may have direct or indirect transmission which occur by way of contaminated food and water (Songer and Post, 2005).

The most likely source of infection for human beings is contact with infected urine from a dog with leptospirosis, especially by owners who retain the pet at home and for hospital personnel who care for the pet during aggressive treatment (Bartges, 2005) and streams and ponds contaminated by the urine of wild rodents or domestic and wild animals can be a source of infection for domestic animals and humans (Songer and Post, 2005).

Leptospires are transmitted between animals by direct or indirect contact. Direct transmission occurs through contact with infected urine, venereal and placental transfer, bite wounds, or ingestion of infected tissues. Recovered dogs excrete organism in urine intermittently for months after infection (Greene *et al*., 2006).

Although transmission of leptospires has traditionally been associated with exposure to infected urine, evidence of its presence was reported in semen and vaginal fluids. Venereal transmission may also occur in small ruminants (Lilenbaum *et al*., 2008).

2.5 Symptoms in dogs

Exposure of susceptible animals to non host adapted serovars of leptospires caused accidental or incidental disease with severe clinical signs (Heath and Johnson, 1994).

Adamus *et al.* (1997) observed that sixteen juvenile Beagle dogs originating from a single breeding colony and regularly vaccinated against *Leptospira interrogans* serogroup canicola and icterohaemorrhagiae developed a clinical syndrome characterized by retarded growth, weight loss and often ascites.

Proteinuria was common in leptospirosis and 27 per cent dogs showed microscopic haematuria and the most common clinical presentation was acute renal failure (Birnbaum *et al.,* 1998).

Three cases of leptospirosis in dogs with serological evidence of infection with *L. interrogans* serovar Pomona and had a common history of anorexia, vomiting, lethargy, and reluctance to walk (Kalin *et al*., 1999).

Boutilier *et al*. (2003) in a retrospective study from dogs with leptospirosis found 67 per cent of dogs had signs of renal failure without evidence of hepatic disease, 20 per cent cases presented with signs of hepatic disease with normal renal function, and 13 per cent cases presented with signs of both renal failure and hepatic disease. The data presented indicated that leptospirosis should be considered as a differential diagnosis for animals presenting with liver disease in the absence of renal failure.

Klaasen *et al*. (2003) challenged dogs with *L. canicola* and *L. icterohaemorrhagiae*, wherein *L. canicola* generally induced a more evident increase in temperature in the controls group of dogs than in vaccinates and after challenge with *L. icterohaemorrhagiae* there was a sharp decrease of the rectal temperature in the control group on day five post-challenge.

Schreiber *et al*. (2005) on experimental infection in dogs with *L. icterohaemorrhagiae* in control and vaccinated group of puppies two weeks after vaccination, only one dog showed macroscopic changes i.e. a widespread icterus, petechiae and icteric discoloration of liver. This dog also showed histological changes in both liver (mild and diffuse congestion) and kidney (diffuse subacute nephritis).

Goldstein *et al.* (2006) in a retrospective study observed that *Leptospira* serogroup pomona caused more severe renal disease and was associated with a worse outcome compared with disease caused by other serogroups.

Geisen *et al*. (2007) found most common presenting clinical signs in leptospirosis in dogs included lethargy, anorexia, vomiting, weakness, and diarrhoea and weight loss. Physical examination abnormalities included icterus (45 per cent), dehydration (31 per cent), red- to brownish-coloured urine (31 per cent) and abdominal pain (19 per cent). Only 36 per cent dogs were febrile (>39^{o} C) and seven (17 per cent) suffered from hypothermia.

Clinical signs of leptospirosis in dogs were non-specific and included jaundice, vomiting, lethargy, abdominal pain and diarrhoea. All the dogs showed multiorgan disease pattern including renal failure and cholestatic hepatopathy (Miller *et al*., 2007).

Minke *et al.* (2009) vaccinated groups of dogs twice with a commercial bacterin containing *Leptospira interrogans* serovars Icterohaemorrhagiae and Canicola and challenged with heterologous representatives of both serovars. Some of these animals were vomiting, slightly dehydrated, and had haematuria. In some dogs, the sclera, gingival and subcutaneous tissues were jaundiced. They also had foul smelling bloody diarrhoea. Renal, hepatic and haematological signs in the clinical presentation of leptospirosis in dogs supported the poly systemic nature of *Leptospira* infection and leptospiraemia persisted for up to six and ten days for *L. interrogans* serovars Icterohaemorrhagiae and Canicola, respectively.

2.6 Clinical pathology

2.6.1 Haematology

Prescott *et al.* (1991) after evaluation of complete blood count (CBC) of a leptospirosis affected dog revealed a mild, non regenerative anaemia which was

presented to a veterinary hospital with acute renal failure and evidence of hepatic insufficiency.

Birnbaum (1998) in a retrospective study on leptospirosis in dogs observed a mild non regenerative anaemia in 33 per cent of dogs and leucocytosis characterized by a mature neutrophilia in 31 per cent of dogs.

Adin and Cowgill (2000) in a retrospective study reported that on haematological examination of 36 dogs with leptospirosis, 55 per cent dogs had neutrophilia and thrombocytopenia.

Boutilier *et al*. (2003) observed 26.6 per cent leptospirosis in dogs had an elevated total WBC count (22.4 to 35.4 × 10^9/L), all of which were characterized as mild to moderate mature neutrophilia and thrombocytopenia was not reported in any of the cases in the present study.

In dogs experimentally challenged with two *Leptospira* strains in control groups, both strains induced a generalised infection in control dogs; the canicola strain was more virulent. Also a statistically significant increase in the percentage of monocytes between days one and six was observed in control groups (Klaasen *et al*., 2003).

Zaragoza *et al*. (2003) observed anaemia (Packed cell volume, 32 per cent) and leucocytosis (maximum, 22.3 × 10^3 cells/µl; minimum, 18.5 × 10^3 cells/µl) in dogs naturally infected with leptospirosis.

Haemato-biochemical analysis should be conducted along with other tests for the confirmatory diagnosis of leptospirosis (Das *et al*., 2004).

Haematological changes in experimental dogs revealed normocytic anaemia, leucocytosis and thrombocytopenia in post inoculation days. Decrease in RBC and haemoglobin concentration in leptospirosis in experimental dogs suggestive of normocytic anaemia might be due the haemorrhage and blood loss in the disease process. Changes on differential leucocytic counts in experimental

dogs revealed a significant ($P<0.05$) increase in neutrophil and monocytic count and also decrease in lymphocyte and eosinophilic counts (Jana *et al*., 2004).

Schreiber *et al*. (2005) in an experimental infection in dogs with *L. icterohaemorrhagiae* in control and vaccinated group of puppies after 2 weeks of vaccination, significant haematological changes were recorded in three successive days (5, 6 and 7) including severe leucocytosis (23,700, 27,900 and 28,900 cells/mm^3) and severe thrombocytopenia (65,000, 132,000 and 158,000 cells/mm^3).

Yang *et al*. (2006) opined that the accelerated platelet clearance by the platelet aggregation and Kupffer cells phagocytosis might be the potential causes of thrombocytopenia in severe leptospirosis rather than diminished production.

Geisen *et al.* (2007) from a complete haemogram of 42 dogs with leptospirosis found 45 per cent dogs showed anaemia with a haematocrit of less than 33 per cent. The white blood cell count was increased (>16 x 10^9/l) in 81 per cent dogs. The leucogram was characterized by neutrophilia with left shift in 25 dogs (>1 x 10^9/l banded neutrophils). Twenty one dogs showed a thrombocytopenia ranging between 8 and 167,000 x 10^9/l. There was no difference in the clinical picture and the laboratory findings between dogs whose sera were reactive to different serogroups.

Claus *et al.* (2008) from seven clinical cases of dogs diagnosed with leptospirosis found leucocytosis in all the affected dogs and two were thrombocytopenic (range 34 x 10^3/µl and 120 x 10^3/µl). Also one dog showed severe anaemia (PCV = 18 per cent).

Jamshidi *et al.* (2008) reported laboratory abnormalities of a clinical case of leptospirosis in a dog which included leucocytosis, thrombocytopenia, haemoconcentration (PCV = 60 per cent), azotemia and high liver enzyme activities (ALT 250 U/L).

In a study of forty seven dogs with leptospirosis, all the clinical groups exhibited a profound anaemia with reduced mean haemoglobin, PCV and RBC. Also all clinical groups exhibited a profound leucocytosis with a prominent neutrophilia and lymphopenia. No significant difference of haemograms within the *Leptospira* serogroups observed (Chandrasekaran *et al.*, 2009).

Minke *et al.* (2009) found on experimental infection of control groups of dogs with *Leptospira interrogans* serovars Icterohaemorrhagiae and Canicola, 40 per cent to 75 per cent of the dogs became thrombocytopenic after challenge, while vaccinated dogs were protected against thrombocytopenia.

2.6.2 Biochemical changes

Prescott *et al.* (1991) found increased urea, creatinine, phosphate, alkaline phosphatase (ALP), and creatinine phosphokinase (CK) from a dog presented with leptospirosis.

Birnbaum *et al*. (1998) documented an increased liver enzyme activity in 61 per cent dogs with leptospirosis.

Adin and Cowgill (2000) in a retrospective study reported that on biochemical examination of 36 dogs with leptospirosis serum biochemical estimations revealed elevated levels of serum creatinine, blood urea nitrogen (BUN), alanine amino transferase (ALT) and alkaline phosphatase (ALP). All leptospirosis affected dogs were initially azotemic. Mean serum creatinine concentration was 7.5 ± 5 mg/dl and mean ALT activity was 161 ± 504 U/L where as 22 per cent dogs were hyperbilirubinemic.

Boutilier *et al.* (2003) observed 80 per cent of the leptospirosis dogs presented with azotemia (Creatinine range = 4.1 to 16.0 mg/dl) and 30 per cent of the dogs had marked elevations in liver enzymes ALP [220 U/L], ALT [174 U/L], and gamma glutamyl transferase (GGT) [22 U/L]).

Zaragoza *et al*. (2003) observed, in dogs naturally infected with leptospirosis serum biochemistry showed normal concentrations of calcium, sodium, potassium and total protein. And some dogs (30 per cent) showed increased concentrations of phosphorus and cholesterol. Decreased concentrations of serum albumin were noticed in 40 per cent dogs with leptospirosis.

Significant serum biochemical changes in experimental dogs infected with leptospirosis were increased total proteins, decreased albumin, elevated ALP, ALT, urea and creatinine (Jana *et al*., 2004).

Schreiber *et al*. (2005) in an experimental infection in dogs with *L. icterohaemorrhagiae* in control and vaccinated group of puppies after 2 weeks of vaccination, a sharp increase in urea (9.4 g/l), creatinine (62 mg/l) and total bilirubin (123 mg/l) were observed on day 7 in one control group puppy.

Geisen *et al.* (2007) in a study of leptospirosis affected cases found increased serum ALT activity (>60 IU/L) in 74 per cent dogs and hyperbilirubinemia (>3.4 µmol/l) in 79 per cent dogs. Azotemia was present in 57 per cent dogs (Creatinine concentration >106 µmol/l).

Azotemia, hypercreatinemia, elevated ALT, ALP, AST and hyperbilirubinemia were evident in leptospirosis (Miller *et al*., 2007).

Claus *et al.* (2008) described seven clinical cases of dogs diagnosed with leptospirosis were initially azotemic and most of the affected cases had an elevated serum AST, ALT and ALP. Hypoproteinemia was present in two out of five dogs, and hypoalbuminemia in two out of six dogs.

Chandrasekaran *et al*. (2009) in a study of forty seven dogs with leptospirosis recorded elevated mean values of BUN, creatinine, ALT, ALP, GGT, total bilirubin as compared to control. No significant difference was noticed within the serogroups.

On experimental infection of control groups of dogs with *Leptospira interrogans* serovars Icterohaemorrhagiae and Canicola, resulted in sharp increases in urea, creatinine, bilirubin, SGOT, or SGPT values after challenge (Minke *et al*., 2009).

2.7 Ultrasonographic findings

Prescott *et al.* (1991) reported abdominal ultrasound of the kidneys and liver were normal of a dog in acute renal failure with evidence of hepatic insufficiency which was diagnosed as a case of leptospirosis.

Bilateral nephromegaly, hyper echoic renal cortices and mild renal pelvis dilation, on abdominal ultrasound of dogs with leptospirosis (Birnbaum *et al*., 1998).

Forrest *et al.* (1998) conducted abdominal ultrasound examinations of 20 dogs with confirmed leptospirosis were reviewed retrospectively for renal abnormalities. Three dogs were normal on ultrasound examination. The remaining 17 dogs had sonographic abnormalities of the kidneys seen either alone or in combination, included renalmegaly (n = 10), pyelectasia (n = 9), increased cortical echogenicity (n = 15), perinephric effusion (n = 5), and a medullary band of increased echogenicity (n = 6).

Abdominal ultrasonographic examination was non significant in a dog presented with leptospirosis (Kalin *et al*., 1999).

On abdominal ultrasonography of 36 dogs with leptospirosis, renal architecture and echogenicity was normal in 78 per cent dogs and mild increase in cortical echogenicity was noted in 22 per cent dogs. Ascites and splenic congestion were evident in 44 per cent dogs (Adin and Cowgill, 2000).

Chandrasekaran *et al*. (2007) observed that the medullary band and medullary rim signs were the specific sonographic findings for leptospirosis in dogs.

Claus *et al*. (2008) on evaluation of abdominal ultrasound in six dogs with leptospirosis observed hyper echoic kidneys in four cases, one of which displayed a dilatation of the renal pelvis. The renal architecture and echogenicity were considered normal in two dogs.

Prathapan (2009) in a study on ultrasonographic changes in fifty dogs with positive MAT titre for leptospirosis revealed hyper echoic liver, thickened gall bladder, portal vein engorgement and few cases exhibited ascites. The nephrosonographic findings included hyper echoic cortex and cortico-medullary distinction was either clear or absent in some cases. He also recorded medullary band, due to increased echogenicity of the medulla in *L. grippotyphosa and L. pomona* infected animals and observed hyper echoic medullary rim as specific sonographic signs for leptospirosis. Nephrosonography was found to be useful in evaluating the extent of tissue damage and in detecting early morphological changes.

2.8 Urinalysis

Urinalysis of a leptospirosis dog revealed a specific gravity of 1.019 with haematuria and proteinuria. Leucocytes (1-4 per high power field) and renal epithelial cells (0-3 per high power field) were observed in the urine sediment (Prescott *et al*., 1991).

Birnbaum *et al*. (1998) observed proteinuria as a common finding on urinalysis of 33 dogs with leptospirosis. Hyposthenuria or isosthenuria noted in 14 out of 19 dogs had concurrent azotemia. Glucosuria was detected in nine per cent dogs and 27 per cent had microscopic haematuria.

Kalin *et al*. (1999) reported urine specific gravity of three dogs affected with leptospirosis was 1.010 – 1.020 and the low urine specific gravity were consistent with renal failure. Also found glucosuria in all the cases and haematuria in two cases.

Adin and Cowgill (2000) performed urinalysis of 36 leptospirosis cases in dogs and found most dogs were isosthenuric and urine specific gravity was <1.020 in 23 out of 36 dogs. Haemoproteinuria was detected in 83 per cent dogs and 17 per cent dogs had microscopic evidence of haematuria. Glucosuria was detected in 26 per cent dogs.

Out of 15 dogs with leptospirosis, 12 dogs presented with azotemia (Creatinine range = 4.1 to 16.0 mg/dl) and all of these dogs had concurrent isosthenuria or hyposthenuria with urine specific gravity ranging from 1.005 to 1.012 (Boutilier *et al.*, 2003).

Zaragoza *et al*. (2003) determined the urine protein pattern in leptospirosis, based on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on the urine, showed 36.7 per cent increase in the excretion of low molecular weight proteins in dogs with leptospirosis but almost no change in the high molecular weight protein excretion.

Seventy one per cent of dogs with leptospirosis showed haematuria and proteinuria in a retrospective study (Geisen *et al*., 2007).

Claus *et al*. (2008) described seven clinical cases of dogs with leptospirosis. Three dogs had isosthenuria (USG = 1.007- 1.015) and proteinuria was detected in three dogs. Two dogs had glucosuria with normoglycemia. Haemoglobinuria and haematuria were found in one dog each.

Urinalysis of a dog with leptospirosis showed proteinuria, and high bilirubin concentration (0.8 mg/dl) along with the presence of large numbers of leucocytes and erythrocytes (Jamshidi *et al.*, 2008).

Okewole and Ayoola (2009) found the urine specific gravity was between 1.004 to 1.010 in 90.4 per cent leptospirosis dogs and only 9.6 per cent dogs retained their urine concentrating ability with values ranging from 1.022 to 1.024. Analysis of urine by dipstick method revealed, +++ occult blood in 61.5 per cent

cases, bilirubin + in 51.9 per cent cases, and a trace of protein in 11.5 per cent cases.

2.9 Pathology

Prescott *et al.* (1991) on histological examination of a dog with leptospirosis revealed renal lesions of sub acute, non suppurative tubulointerstitial nephritis. The tubular epithelium had undergone focal degeneration characterized by cellular swelling, karyolysis, and loss of cells into the lumen to form cellular casts. Focal accumulations of inflammatory cells were present within the interstitium between the tubules.

Adamus *et al.* (1997) studied sixteen juvenile Beagle dogs on post-mortem gross lesions were confined to the liver which was often firm, tan coloured and mottled. Microscopically hepatic lesions ranged from those of severe chronic hepatitis to mild diffuse hepatocellular vacuolation, with bile stasis. This study has established an association between spontaneous chronic hepatitis and intralesional leptospires in dogs.

Boqvist *et al*. (2003) based on the immunofluorescence, pathological and serological findings on fattening pigs in the Mekong delta suggested that the presence of macroscopic renal lesions (white spots) was not a reliable indicator of the presence of leptospires in the kidneys.

Infection with *Leptospira canicola* in control group of dogs caused histopathological evidence of interstitial nephritis (Klaasen *et al*., 2003).

Zaragoza *et al.* (2003) based on the urine protein pattern in leptospirosis by performing sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on the urine viewed that canine leptospirosis was characterized by interstitial nephritis.

Greenlee *et al*. (2004) experimentally inoculated two strains of *L. kirschneri* serovar Grippotyphosa in dogs and based on the evaluation of

necropsy lesion and clinicopathologic data concluded that, infection with these two strains resulted in severe renal and hepatic damage.

Faria, *et al*. (2007) conducted a histopathological analysis of kidney tissues from wild captured and experimentally infected rats and concluded that interstitial nephritis was the only lesion attributable to leptospiral infection.

Minke *et al*. (2009) on experimental infection of control groups of dogs with *Leptospira interrogans* serovars Icterohaemorrhagiae and Canicola, found prominent lesions in the kidneys of terminally ill control dogs which included sub acute to severe interstitial glomerulo-nephritis and tubular degeneration. Moderate to severe diffuse hepatic lesions were found in dogs with jaundice, mostly consisting of an acute degenerative hepatitis characterized by hepato-cellular dissociation and necrosis.

2.10 Diagnosis

2.10.1 Antigen detection

2.10.1.1 Dark field microscopy (DFM)

Prescott *et al*. (1991) suggested dark field microscopy was not always a diagnostically rewarding procedure on clinical specimens as shedding of leptospires could be intermittent.

Chandrasekaran and Pankajalakshmi (1997) observed that dark field microscopy after differential centrifugation of urine was useful in the early diagnosis of leptospirosis.

Chandrasekaran *et al*. (1998) in a study observed 82 per cent panuveitis cases showed the presence of *Leptospira* in their blood samples by dark field microscopy.

Kalin *et al*. (1999) described three cases of dogs with positive antibody titre against serovar pomona but dark field microscopy did not reveal the presence of leptospires in two cases.

Vijayachari *et al*. (2001) reported DFM had a sensitivity of 40.2 per cent, specificity of 61.5 per cent, a positive predictive value of 55.2 per cent and a negative predictive value of 46.6 per cent and did not recommend DFM as a sole diagnostic procedure for early diagnosis of leptospirosis.

Zaragoza *et al.* (2003) demonstrated *L. interrogans* microscopically in the urine of all ten dogs showing clinical symptoms suggestive of leptospirosis.

Elaiyaraja (2003) demonstrated the presence of leptospires in five blood samples and one urine sample by DFM among the samples (22 blood and 5 urine samples) collected from dogs. Of the 30 urine samples collected from bovines, none were found to be positive by DFM.

Dhannia (2005) reported on examination of 68 blood samples, two blood samples were DFM positive, but later PCR and culture detected both the samples as negative for leptospires.

Magajevski *et al*. (2005) could not observe any leptospires by the direct dark field microscopy in the semen samples as well as urine samples collected from serologically reactive bulls.

Dark field examination was necessary for rapid identification of viable leptospires because they cannot be stained by simple methods with aniline dyes. But a variety of bacteria that could be confused with leptospires produce more random movement in wet mount preparations and also cellular fibrils and fibrin strands can be mistaken for organisms (Greene *et al.*, 2006).

Kuriakose *et al.* (2008) recorded positive result only from three urine samples out of 54 rats urine examined by dark field microscopy.

Sharma and Kalawat (2008) reported that specificity and sensitivity of dark field microscopy for detection of leptospires in blood sample were 61 per cent and 60 per cent respectively. They also recommended dark field microscopy, when other diagnostic tools were not readily available.

Lilenbaum *et al*. (2009) observed leptospires by DFM in eight urine samples from 59 animals originated from goat and sheep. They suggested direct visualization of leptospires in fresh specimens by DFM was very difficult and could not be considered as a reliable method for detecting carriers.

Shivaraj *et al.* (2009) in a study of total 70 serum/blood samples collected from dog population in and around Bangalore were subjected to DFM and PCR for detecting leptospires. In all the samples *Leptospira* like organisms were not detected by DFM and none of the samples produced an amplicon by PCR indicating that leptospires were not detected in the samples screened. But MAT showed significant antibodies titres in few sera samples against serovars australis, pyrogenes and autumnalis which might be due to past exposure to the infection.

2.10.1.2 Staining

Prescott *et al.* (1991) demonstrated large numbers of typical leptospires against serovar pomona in urine sample by immunofluorescence staining.

Boqvist *et al*. (2003) demonstrated leptospires in 22 (69 per cent) of the investigated kidneys by immunofluorescence in fattening pigs.

Jamshidi *et al.* (2008) by silver impregnation technique using Levaditi's stain demonstrated spirochetes inside of hepatocytes and in sinusoids.

2.10.1.3 Isolation

Boqvist *et al*. (2003) could isolate *Leptospira interrogans* serovar Bratislava from kidney of a fattening pig by culture.

Magajevski *et al.* (2005) in relation to the results of hamster inoculation with freshly collected semen observed that the passage of material in laboratory animals could be a good alternative for isolation of *Leptospira* species from semen.

Jamshidi *et al.* (2008) could isolate the leptospires after inoculating the fresh urine sample in to Fletcher's culture media.

2.10.2 Antibody detection

2.10.2.1 Agglutination tests

2.10.2.1.1 Microscopic Agglutination Test (MAT)

MAT titres in excess of 1:400 or a four-fold rise in the titre in paired samples are diagnostically significant when accompanied by clinical signs consistent with leptospirosis. Serological diagnosis of host-adapted leptospirosis is difficult as titres may by decreasing or absent when clinical signs are observed (Quinn *et al*., 2002).

A result of 50 per cent agglutination of leptospires at 1:100 dilutions was considered significant. i.e., the serum samples having titres greater than or equal to 100 were considered positive (Dey *et al*., 2004).

The MAT proved to be the most specific test which could also identify the serogroup identity of the infecting *Leptospira* (Soman, 2004).

Lilenbaum *et al.* (2009) considered the combined use of MAT as a screening test followed by urine PCR for the direct detection of *Leptospira* spp. DNA was adequate for the identification of carrier animals among goats and sheep.

Jamshidi *et al.* (2008) found positive MAT titre by 1:400 dilutions against *L. canicola* from a confirmed case of leptospirosis in dog.

2.10.2.1.2 Latex Agglutination Test (LAT)

LAT was extremely rapid (2-10 min), simple and inexpensive test which was well within the capability of most laboratories. Also it was sensitive in detecting leptospiral antibodies and could be a useful screening test for detecting leptospiral antibodies (Ramadass *et al*., 1999).

A rapid recombinant LipL32 antigen-based latex agglutination test (LAT) had been developed to detect specific antibodies of the pathogenic serovars of *Leptospira* in the acute phase of the illness from human and dog sera as the expression of the LipL32 antigen was restricted only to the pathogenic leptospires. The test was found to be sensitive, specific and accurate as compared to the standard microscopic agglutination test (Dey *et al*., 2007).

The sensitivity and specificity of the LAT were 89.7 and 9.45 per cent in rapid serodiagnosis of leptospirosis (Senthilkumar *et al*., 2008).

2.10.2.2 Enzyme Linked Immunosorbent Assay (ELISA)

Dhaliwal *et al*. (1996) after examining samples of cervico-vaginal mucus and post calving discharges from cows naturally infected with *Leptospira interrogans* serovar Hardjo opined that the serum IgG-ELISA was the most efficient in detecting hardjo antibodies, but the IgG- and IgA-ELISA of the post calving discharge proved to be equally effective.

Soman (2004) after evaluation of three diagnostic tests, Indirect ELISA was found to be most sensitive for rapid screening of leptospirosis.

2.10.3 Polymerase Chain Reaction (PCR)

PCR had proven to be specific for leptospirosis, more sensitive and more rapid than culturing, and in contrast to serology, informative in the first week of illness. Detection of leptospires in urine with PCR was a promising approach for

early diagnosis of leptospirosis and might also be useful in studying long term shedding (Bal *et al.,* 1994).

Positive polymerase chain reaction (PCR) test results prior to seroconversion were beneficial in establishing an early diagnosis of leptospirosis in dogs (Harkin *et al*., 2003).

Elaiyaraja (2003) found the PCR technique to be more sensitive, specific and rapid over conventional methods as it detected 41.6 per cent, compared to 25.6 per cent by dark field microscopy and 2.4 per cent by culture of the samples tested.

Shukla *et al*. (2003) reported a newly identified region of 16S rRNA gene for rapid and distinct identification of an isolate in terms of its pathogenic potential compared to the conventional tests. Amplification at 1.1 kb region identified the isolate to the genus level and presence of second band at 630 bp provided pathogenic identity.

Dhannia (2005) reported that molecular methods were ideal in differentiating *Leptospira* serovars in biomaterials. Among the different PCR technique used in study LS- PCR (low stringency) was found to be more useful which could group the 16 leptospires into 10 groups in one step analysis.

Multiplex PCR assay would be very cost effective and rapid diagnostic methods for canine parvo viral infection and leptospirosis when concurrent infections were suspected in dogs (Ramadass and Latha, 2005).

Meenambigai *et al.* (2006) could detect twelve serogroups of pathogenic *Leptospira interrogans* species by polymerase chain reaction using G1 and G2 primers.

Nested PCR was a rapid, specific and sensitive tool that could aid in the detection of *Leptospira* spp. in bovine urine samples from herds with a clinical suspicion of leptospirosis (Bomfim *et al*., 2008).

Sensitivity of PCR often precludes the need for isolation and culture, thus making it ideal for the detection of organisms involved in acute infections (Lilenbaum *et al*., 2009).

2.11 Treatment

Haemodialysis had been shown to improve the prognosis of dogs with leptospirosis and severe azotemia (Adin and Cowgill, 2000).

During canine leptospiraemia, antibiotics of choice were penicillin G, ampicillin, or amoxicillin, with dosages adjusted for degree of renal insufficiency. Doxycyline was often used in dogs especially during leptospiruria (Songer and Post, 2005).

Greene *et al*. (2006) suggested specific therapy for leptospirosis consisting of antibiotics which minimizes organ damage and quickly clears the leptospiraemic phase. Penicillin and its derivatives were the antibiotics of choice for eliminating leptospiraemia, but they did not eliminate the carrier state. Doxycycline also could be used for initial therapy or for elimination of the carrier state in leptospirosis.

After oral administration, urine concentrations of doxycycline in normal cats and dogs were sufficient to inhibit the growth of significant number of urinary tract pathogens (Wilson *et al*., 2006).

Geisen *et al.* (2007) reported a survival rate of 52 per cent in leptospirosis affected animals.

Claus *et al.* (2008) administered amoxicillin or a combination of amoxicillin with clavulanic acid (8.5 mg/kg s.c. q 12 h) to all seven clinical cases of dogs diagnosed with leptospirosis for two weeks. Five of the seven dogs in this study made complete recovery with regard to renal function. He recommended treating every patient with clinical signs and laboratory

abnormalities of acute renal failure without a known cause with penicillin derivatives until specific testing confirms or rules out leptospirosis.

2.12 Immunity

Klaasen *et al*. (2003) demonstrated, after two vaccinations with a commercial canine leptospirosis vaccine, duration of immunity of one year and all vaccinated dogs were protected for 13 months from renal infection with *Leptospira canicola.*

A cross-protective effect with pathogenic strains of *Leptospira* was shared by Hemolysin-Associated Protein 1 (Hap1) mediated by a DNA plasmid vector. This could be helpful for designing and development of a new generation of vaccines against bacteria, particularly *Leptospira interrogans* sensu lato (Branger *et al*., 2005).

Schreiber *et al*. (2005) on immunisation with a bivalent *Leptospira* vaccine showed to protect dogs against symptomatology and to prevent leptospiraemia, urine shedding and the renal infection.

Soto *et al*. (2008) observed passive immunity induced by commercial polyvalent whole-bacteria bacterins was short duration and unable to confer protection against leptospirosis in newborns.

A primary course of two doses of vaccine containing *Leptospira interrogans* serovars Icterohaemorrhagiae and Canicola provided quick onset and long-term protection against both clinical leptospirosis and the renal carrier stage (Minke *et al*., 2009).

Materials and Methods

3. MATERIALS AND METHODS

The study was conducted at the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Mannuthy during the period of Aug 2008 to Mar 2010.

3.1 Materials

3.1.1 Samples

Dogs presented to University Veterinary Hospital Mannuthy and Kokkalai with signs suggestive of leptospirosis formed the study material. Blood and urine samples were examined for leptospires under dark field microscopy which were preserved at -20^{o} C until use and microscopic agglutination test and polymerase chain reaction were performed on the suspected samples.

Signalment and history of the cases were noted. Detailed examinations were made on each case. Haematology and biochemical tests were done and all the cases were subjected to ultrasonography. To test the significance between the diseased and the control group Student's 't' test was carried out at five per cent level.

Leucocytosis was defined as a total leucocyte count in excess of 12 × 10^3/µl. Normal erythrocyte count of a dog blood was taken in a range of 6-8 million/µl. Thrombocytopenia was defined as a platelet count less than 200,000/µl. Renal failure was defined as azotemia on initial presentation (Creatinine greater than 1.5 mg/dl with low urine specific gravity). Liver parameters were considered markedly elevated if they were more than twice the upper normal limit. Based on presenting complaint and biochemical abnormalities these patients were classified as having primarily either renal or hepatic involvement, or both.

3.1.2 Glassware, Plastic ware and Chemicals

All glasswares used were of Borosil brand. Plastic ware used was of Tarson brand and chemicals were of analytical grade.

3.1.3 Buffer and Reagents

PBS (pH 7.2) – 10 X stock solution

Sodium chloride	80 g
Disodium hydrogen ortho phosphate	12.72 g
Potassium dihydrogen ortho phosphate	2 g
Distilled water	1000 ml

pH was adjusted to 7.2 by adding 0.1 M sodium hydroxide.

Working solution PBS – 1 X

Stock solution	1 ml
Distilled water	9 ml

3.1.4 MAT for antibody detection

MAT was done for detection of *Leptospira* agglutinating antibodies at Leptospira Research Laboratory, Centre for Animal Health Studies, Madhavaram Milk Colony, Chennai.

The sera samples which showed 1:100 titre against *Leptospira* serovars other than vaccine serovars like *L. icterohaemorrhagiae, L. canicola, L. grippotyphosa* and *L. pomona* with symptoms suggestive of leptospirosis were taken as positive cases.

3.1.4.1 Serovars used as antigen for MAT

Reference strain	*L. canicola*
	L. icterohaemorrhagiae
	L. autumnalis
	L. australis
	L. ballum
	L. tarassovi
	L. hebdomadis
	L. pomona
	L. pyrogenes
	L. grippotyphosa
	L. hardjo
	L. javanica

3.1.5 Polymerase Chain Reaction

3.1.5.1 Tris EDTA Borate Buffer (TBE) - 10 X

Tris base	108 g
Boric acid	5.5 g
0.5 M EDTA	40 ml

Made up the volume to one litre by adding distilled water. From this10 X buffer, prepared 500 ml of 1 X buffer by adding 50 ml of the buffer to 450 ml of distilled water.

3.1.5.2 Ethidium bromide Stock solution

Ethidium bromide	10 mg
Triple distilled water	1 ml

3.1.5.3 Agarose gel (0.8 per cent) preparation and casting

1. Weighed out 1.2 g agarose in a beaker
2. Added 150 ml of 1 X TBE buffer to the beaker
3. Melted the agarose in the microwave oven (60^0 C for two min)
4. Allowed to cool to handling temperature
5. Five micro litres of ethidium bromide stock solution was added in the gel before it solidifies
6. Placed the comb in position in the electrophoresis tank
7. Poured the prepared gel and allow to solidify for about 30 minutes
8. Removed the comb

3.1.5.4 Gel loading Dye (6 X)

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

3.1.5.5 Preparation of DNA from clinical samples

3.1.5.5.1 QIAGEN, DNA extraction kit (DNeasy Spin-Column)

The DNeasy membrane combined the binding properties of a silica-based membrane with simple microspin technology. Buffer conditions in DNeasy procedures were designed to enable specific adsorption of DNA to the silica membrane and optimal removal of contaminants and enzyme inhibitors.

a) **Buffer ATL**

b) **Proteinase K**

c) AL Buffer

d) Ethanol (96–100 per cent)

e) Wash buffer 1 (AW1)

f) Wash buffer 2 (AW2)

g) Elution buffer (AE buffer)

3.1.5.6 PCR amplification

a) Primers

Primer sequences of pathogenic *Leptospira* specific 16S rRNA gene developed by Microbiology division, DRDE, Gwalior were synthesized by M/S Genetix, New Delhi.

Forward primer 5' CGCTGGCGGCGCGTCTTAAA 3'

Reverse primer 5' AAGGTCCACATCGCCACTT 3'

b) PCR Reaction buffer (10 X)

This includes 500 mM KCl and 100 mM Tris hydrochloride.

c) Magnesium Chloride

Magnesium chloride with strength of 25 mM.

d) *Taq* DNA Polymerase

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

e) Deoxy Ribonucleotide Triphosphates

Deoxy ribonucleotide triphosphates (dNTP) mix 2.5 mM each (10 mM of each dGTP/ dCTP/ dATP/ dTTP in equal volumes).

3.2 Methods

3.2.1 Collection of samples

Blood and serum was collected by puncturing the cephalic or saphenous vein. Potassium Ethylene Diamine Tetra Acetic acid (EDTA) was used as anticoagulant (1 mg/ml of blood).

3.2.1.1 Blood and Serum

About three millilitres of blood samples were collected in sterile vials containing EDTA anticoagulant. About four millilitres 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.

3.2.1.2 Urine

Urine was collected aseptically from dogs by catheterization. Infant feeding tubes size seven and eight were used for the same depending on the size of the dogs. About fifty micro litres of urine was collected. For DFM examination sufficient quantity of urine taken into equal quantity of sterile phosphate buffered saline (pH 7.2) in sterile vials.

3.2.2 Preservation of samples

Both urine and blood samples were preserved for the purpose of performing polymerase chain reaction to find out the pathogenic leptospires. About two millilitres of serum was used to perform MAT and biochemical tests. Blood and urine were preserved in sterile eppendorf tubes by adding one per cent merthiolate solution @ 20 micro litres per ml of sample and the samples were kept at -20^{o} C until use.

3.2.3 Dark Field Microscopy

3.2.3.1 Blood

Two millilitres of blood samples collected in 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 (10 X) and high (45 X) 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 10,000 rpm for 15 min and the sediment was examined on the same way.

3.2.3.2 Urine

Five millilitres 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 (10 X) and high (45 X) 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 MAT

Procedure

MAT was carried out as per the procedure of Faine *et al*. (2000) using a battery of live leptospira serovars. Test was carried out in micropipette plates with round bottom. A volume of 25µl of sterile phosphate buffer saline (PBS) pH 7.2 was added to each well of the plate except the first row. The sera samples were diluted to 1:50 in PBS in test tubes. Transferred 50 µl of 1:50 diluted serum sample to the first well, making a dilution of 1:50 and then continued serial dilution to 1:3200 by transferring 25 µl. To these wells 25 µl of liquid culture was added. The final dilution after addition of the antigen ranged from 1:100 to

1:6400. Known positive and negative control sera were included. The samples were mixed and incubated at 37° C for two hours. At the end of incubation, 10 µl of each dilution was taken on a clean glass slide and examined under low power (10 X) of dark field microscope, without using a cover slip.

The end point of agglutination reaction was taken as the highest point in which 50 per cent of the leptospires had agglutinated. The reciprocal of end point was taken as the titre. A titre of 100 and above was considered positive (Faine *et al*., 2000).

3.2.5 PCR

3.2.5.1 Preparation of DNA from clinical samples

(DNeasy Blood and Tissue kit)

3.2.5.1.1 Blood

1. Pipetted 20 µl proteinase K into a 1.5 ml microcentrifuge tube. Added 50–100 µl anticoagulated blood. Adjusted the volume to 220 µl with PBS.

2. Added 200 µl Buffer AL (without added ethanol). Mixed thoroughly by vortexing, and incubated at 56° C for 10 min.

3. Added 200 µl ethanol (96–100 per cent) to the sample and mixed thoroughly by vortexing.

4. Pipetted the mixture from step three into the DNeasy Mini spin column placed in a two ml collection tube. Centrifuged at 8000 rpm for one min. Discarded flow-through and collection tube.

5. Placed the DNeasy Mini spin column in a new two ml collection tube, added 500 µl Buffer AW1, and centrifuged for one min at 8000 rpm. Discarded flow-through and collection tube.

6. Placed the DNeasy Mini spin column in a new two ml collection tube, added 500 µl Buffer AW2, and centrifuged for three min at 14,000 rpm to dry the DNeasy membrane. Discarded flow-through and collection tube.

7. Placed the DNeasy Mini spin column in a clean 1.5 ml microcentrifuge tube and pipetted 200 µl Buffer AE directly onto the DNeasy membrane. Incubated at room temperature for one min and then centrifuged for one min at 8000 rpm to elute.

3.2.5.1.2 Urine

1. Pelleted down the cell debris at 2000 – 3000 rpm for 10 – 15 min.

2. Suspension took as sample.

3. Pelleted down the suspension as at 13,000 rpm for 30 – 45 min.

4. Removed the supernatant and took the pellet as bacterial cell.

5. Added 100 µl PBS (1X) and mixed well and centrifuged again at 10,000 rpm for five min.

6. Discarded the supernatant.

7. Added 180 µl Buffer ATL to the pellet and resuspended it by vortexing.

8. Added 20 µl proteinase K. Mixed thoroughly by vortexing, and incubated at 56°C until the tissue was completely lysed.

9. Vortexed for 15s. Added 200 µl Buffer AL to the sample, and mixed thoroughly by vortexing. Then added 200 µl ethanol (96–100 per cent) and mixed again thoroughly by vortexing.

10. Pipetted the mixture from step nine (including any precipitate) into the DNeasy Mini spin column placed in a two ml collection tube. Centrifuged at 8000 rpm for one min. Discarded flow-through and collection tube.

11. Placed the DNeasy Mini spin column in a new two ml collection tube, added 500 µl Buffer AW1, and centrifuged for one min at 8000 rpm. Discarded flow-through and collection tube.

12. Placed the DNeasy Mini spin column in a new two ml collection tube, added 500 µl Buffer AW2, and centrifuged for three min at 14,000 rpm to dry the DNeasy membrane. Discarded flow-through and collection tube.

13. Placed the DNeasy Mini spin column in a clean 1.5 ml microcentrifuge tube, and pipetted 200 µl Buffer AE directly onto the DNeasy membrane. Incubated at room temperature for one min, and then centrifuged for one min at 8000 rpm to elute.

3.2.5.2 PCR amplification

Each 50 µl PCR reaction contained,

Template DNA	2.5 µl
10 X PCR Buffer	5 µl
10 mM $MgCl_2$	7.5 µl
dNTP mix (2.5 mM each)	5 µl
Taq DNA polymerase (3U/ µl)	0.17 µl
Primer P1 (20 pmole)	0.5 µl
Primer P2 (20 pmole)	0.5 µl
Distilled water	28.83 µl

Standard positive controls of leptospiral DNA were also included. The tubes were spun briefly and placed in the thermal cycler.

3.2.5.3 PCR amplification conditions

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

Stage	No. of cycles	Step	Temperature ^{0}C	Time	Purpose
1	1	1	94	5 min	Initial denaturation
2	30	1	94	1 min	Repeat denaturation
		2	55	1 min	Annealing
		3	72	2 min	Extension
3	1	1	72	10 min	Final extension
4	1	1	4	HOLD	Storage

3.2.5.4 Detection of PCR products

3.2.5.4.1 Submarine Agarose gel electrophoresis

Two micro litre of each PCR product was mixed with 6 X gel loading dye and loaded into the wells. Positive controls were also set. The gel was run at 70 V for 30 - 45 minutes. The amplification was observed under transilluminator

(Bio Rad, USA) and was documented using gel documentation system (Gel Doc, Sony, Japan).

Appearance of a band of approximately 630 bp when compared with the DNA ladder was taken as positive.

3.2.6 Evaluation of Haematological Parameters

Parameters viz; Volume of packed red cells (VPRC), haemoglobin (Hb), total erythrocyte count (TEC), platelet count, total leucocyte count (TLC), differential leucocyte count (DLC), were estimated by using the BV – 4100 FULLY AUTOMATED HAEMATOLOGY ANALYSER^{R}.

3.2.7 Serum Biochemistry

Total serum protein, albumin, globulin, AG ratio, creatinine, ALT, total bilirubin and direct bilirubin were estimated by spectrophotometry in MERCK MICROLAB – 200^{R} by using Agappe reagents.

Serum total protein was estimated by Direct Biuret method described by Gomall *et al.* (1949) whereas serum albumin was estimated by Bromocresol green methodology described by Doumas *et al*. (1971). Serum globulin was calculated from the difference of serum total protein and albumin (Benjamin, 1985). Albumin / globulin ratio (A/G ratio) was calculated from the albumin and globulin values (Benjamin, 1985). Total and direct bilirubin was estimated by modified DMSO method described by Walter and Gerard (1980) and serum creatinine was estimated by modified Jaffe's method as described by Allen *et al*. (1982).

3.2.8 Ultrasonography

Dogs were subjected detailed abdominal ultrasound scanning using HS – 2000 VET, Honda Electronics to identify the presence of any kind of organ abnormalities in kidney and liver.

3.2.9 Urinalysis

Urine specific gravity was determined by urinometer method. Occult blood by Benzidine test. Protein by Heller's test. Bile pigment by Fouchet's test. Bile salt by Hay's test. Glucose by Benedict's test, as described by Benjamin (1985).

Urinalysis was also done with commercially available urine strip – Vet Uro Color^{R} by Anigen, Korea when the urine quantity was less.

3.2.9 Treatment

Positive cases divided into two groups. Group I treated with Amoxicillin–Clavulanic acid @ 12.5 mg/kg.b.wt. at 12 hr interval parenterally for 7 days and Group II treated with Benzyl penicillin @ 50,000 IU/kg b.wt. at 12 hr interval, parenterally for 7 days along with supportive therapy to manage the clinical condition.

Results

4. RESULTS

The dogs presented to University Veterinary Hospitals, Mannuthy and Kokkalai with symptoms suggestive of leptospirosis were screened by DFM, MAT and PCR.

4.1 Examination of Leptospires in suspected cases

4.1.1 Dark Field Microscopy

4.1.1a Blood

Blood samples from twenty dogs showing symptoms suggestive of leptospirosis were examined by DFM, after centrifugation. No blood samples revealed presence of typical, motile leptospires.

4.1.1b Urine

Urine samples of all the suspected cases taken for DFM examination after centrifugation, revealed no leptospires.

4.1.2 MAT

The MAT titres of samples are summarized in Table 1.

Out of the 20 sera samples tested 12 were positive for antibodies to *Leptospira* serovars (Plate 1 and 2).

The sera samples showed a mixed type of agglutinating antibodies with different leptospiral serovars. Predominant seroreactions were to *L. autumnalis* and *L. australis* in nine cases (75 per cent each). Four sera samples (33.3 per cent) reacted to *L. icterohaemorrhagiae*, three sera samples each (25 per cent) reacted to *L. javanica*, *L. pyrogenes* and *L. hebdomadis*. Two sera samples (16.6 per cent) reacted to *L. canicola* and one serum sample (8.3 per cent) reacted to *L. grippotyphosa*.

Among seropositive dogs six cases (50 per cent) reacted at a higher titre of 1:400 or more, and remaining six cases reacted to low titre of 1:100.

Case no. 8724 showed 1:800 titres against *L. grippotyphosa* and 1:400 against *L. icterohaemorrhagiae* and was taken as positive case.

One serum sample (Case no. 7479) had showed a high titre of 1:3200 against *L. icterohaemorrhagiae, L. hebdomadis* and *L. javanica.*

4.1.3 PCR

PCR was carried out to amplify 630 bp pathogenic specific 16S rRNA gene segment from blood and urine samples of suspected cases of leptospirosis. Out of 20 samples each of blood and urine tested, one sample of blood and one sample of urine were found to be positive for the pathogenic leptospires. In the positive samples, an approximately 630 bp amplified products could be observed (Plate 3).

4.2 History and Clinical manifestation of leptospirosis in dogs

Among 12 positive cases, five were males and seven were females (Table 2 and Fig. 1). Most presented breeds were Labrador retrievers (Four cases), German shepherd, Rottweilers (Two cases each), Spitz, Dachshund (One case each) and two cases of non descriptive dogs respectively (Table 3 and Fig. 3). Seven cases were within an age group of one to three years, and remaining five were in an age group between three to six years (Table 4 and Fig. 2).

On analysing clinical manifestation of positive cases, animals were presented with inappetence (58.33 per cent), anorexia (41.67 per cent), vomiting, jaundice (58.33 per cent each) (Plate 4 and 5), haematuria, haemoglobinuria (25 per cent each) and melaena (25 per cent) (Table 5 and Fig. 4). Temperature was within the normal range of 101 – 103^{o} F in 75 per cent cases. Only one case showed 104^{o} F. Two cases showed a slight decrease in temperature (100^{o} F). The cases were presented mostly in monsoon months (July to November - 9 cases).

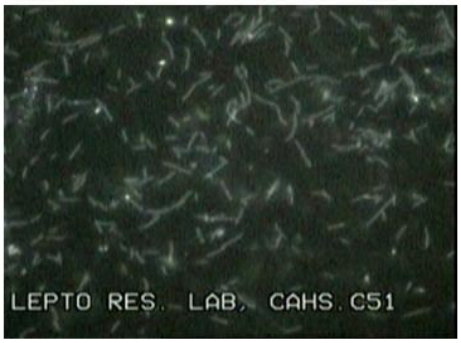


Plate 1 - Culture of leptospires under DFM (200 x)

Plate 2 - Agglutination of leptospires in MAT (50 per cent reaction) 200 x

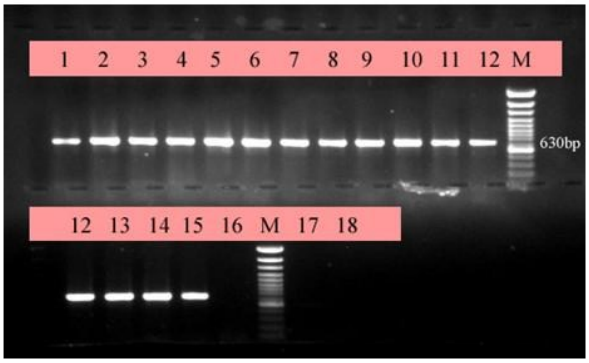


Plate 3 - PCR assay specific for pathogenic leptospires, based on 16 S rRNA gene, showing approximately 630 bp amplified product

1-australis, 2- autumnalis, 3- ballum, 4- bataviae, 5- canicola, 6- cynopteri, 7- djasmin, 8- grippotyphosa, 9- hardjo, 10- hebdomadis, 11- pomona, 12- sarmin, 13- shermani,14 - C361 Urine, 15 - C951 Blood, 16 - C951 Urine, 17 - C361 Blood, M-100bp Marker,18 - C8773 Urine, 19 - C8773 Blood

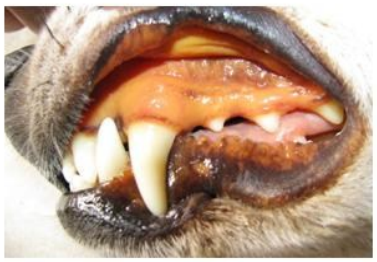


Plate 4
Icteric oral mucous membrane of dog

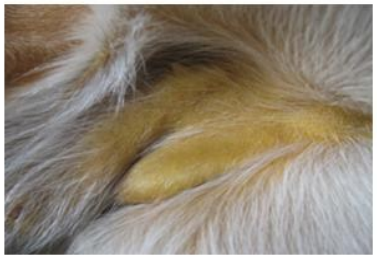


Plate 5
Icteric ventral abdomen of dog

Table 1. MAT titre of samples

SI No.	Case no.	*L. icterohaemorrhagiae*	*L. canicola*	*L. grippotyphosa*	*L. hebdomadis*	*L. pomona*	*L. autumnalis*	*L. pyrogens*	*L. tarassovi*	*L. ballum*	*L. javanica*	*L. australis*	*L. hardjo*
1	8773						1:400					1:100	
2	6455						1:100						
3	10366		1:100				1:100						
4	361				1:800						1:400	1:100	
5	7479	1:3200	1:100		1:3200		1:400	1:400			1:3200	1:100	
6	6206						1:100					1:100	
7	962						1:100					1:100	
8	951	1:100			1:400		1:400	1:400			1:400	1:100	
9	8429						1:100					1:100	
10	4800						1:400	1:400				1:400	
11	8724	1:400		1:800									
12	4735	1:100										1:100	

Table 2. Sex wise distribution of *Leptosopira* seropositive dogs

Sex	No. of animals	Per cent
Male	5	42
Female	7	58

Table 3. Breed wise distribution of *Leptosopira* seropositive dogs

Breed	No. of animals	Per cent
Labrador	4	33.33
German shepherd	2	16.67
Rottweiler	2	16.67
Spitz	1	8.33
Dachshund	1	8.33
Non descriptive	2	16.67

Fig. 1 Sex-wise distribution of *Leptospira* seropositive dogs

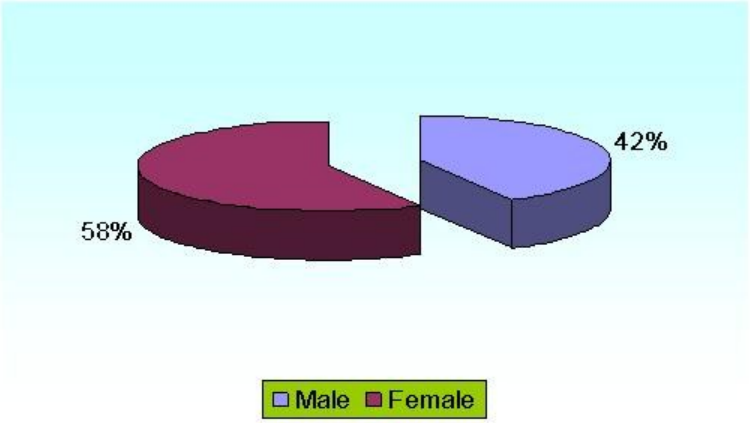


Fig. 2 Age-wise distribution of *Leptospira* seropositive dogs

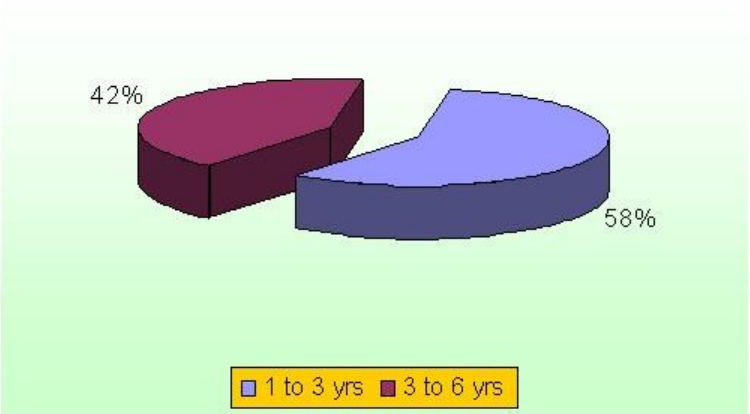


Table 4. Age wise distribution of *Leptosopira* seropositive dogs

Age group (years)	No. of animals	Per cent
One to three	7	58
Three to six	5	42

Table 5. Clinical manifestations in dogs positive for leptospirosis

Clinical signs	No. of positive cases	Per cent
Inappetence	7	58.33
Anorexia	5	41.67
Vomiting	7	58.33
Jaundice	7	58.33
Haematuria	5	41.67
Haemoglobinuria	5	41.67
Melaena	3	25

Fig.3 Breed-wise distribution of *Leptospira* seropositive dogs

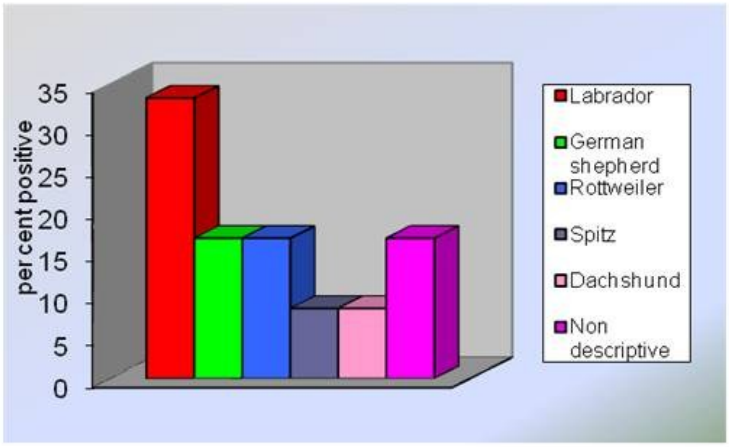
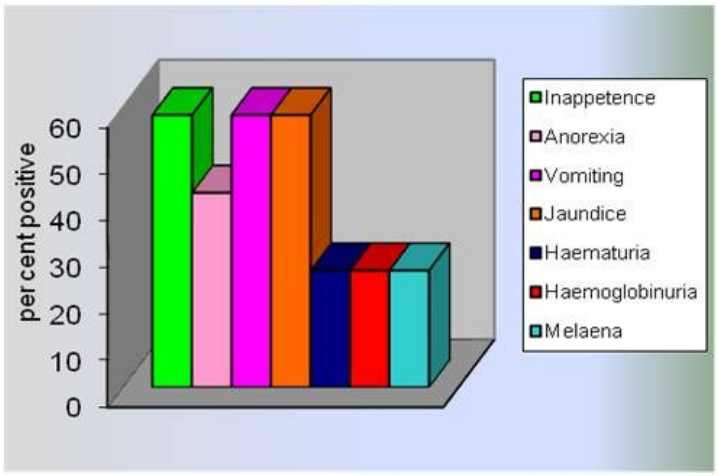


Fig.4 Clinical signs in *Leptospira* seropositive dogs



4.3 Haematology

The mean haematological values of control and diseased animals are presented in Table 6 and haemogram of diseased animals in Table 7.

4.3.1 Total Leucocyte Count and Differential Leucocyte Counts

4.3.1a *Total leucocyte count*

The mean total leucocyte counts were apparently higher in seropositive dogs and the values in positive and control groups were 37.13 ± 8.67 x 10^3 per µl and 10.15 ± 0.61 x 10^3 per µl respectively, which was statistically significant and suggestive of leucocytosis in diseased animals. There was marked increase in total leucocyte count as shown by the most of the diseased animals. About 91.67 per cent (11 out of 12) animals showed a leucocyte count more than 17,000 per µl.

4.3.1b *Neutrophil count*

The difference between neutrophil count between diseased and control group was significant, with mean values at 79.83 ± 2.77 per cent and 71.67 ± 1.52 per cent respectively and suggested neutrophilia.

4.3.1c *Lymphocyte count*

The mean lymphocyte count was 16.67 ± 2.24 per cent and 25.33 ± 1.31 per cent respectively in the diseased and control groups. The mean count was suggestive of slight reduction in lymphocyte counts.

4.3.1d *Eosinophil count*

The mean eosinophil counts in diseased and control dogs were 1.42 ± 0.26 per cent and 1 ± 0.36 per cent respectively. Statistical analysis showed no significant difference.

4.3.1e *Monocyte count*

The mean monocyte counts in diseased and control dogs were 1.25 ± 0.57 per cent and 2 ± 0.26 per cent respectively. Statistical analysis showed no significant difference.

4.3.2 Total Erythrocyte Count

The mean erythrocyte counts in diseased and control dogs were 4.92 ± 0.67 x 10^6 per µl and 6.89 ± 0.11 x 10^6 per µl respectively. The decrease in the mean erythrocyte count was statistically significant in diseased dogs and was suggestive of mild anaemia. Out of twelve animals, five animals showed a normal range of erythrocyte count (6–8 x 10^6 per µl). But remaining animals (58.33 per cent) showed a marked reduction in erythrocyte count, suggestive of anaemia.

4.3.3 Platelet Count

The difference between mean platelet count between diseased and control group was significant, with mean values at 1.91 ± 0.34 x 10^5 per µl and 3.67 ± 0.11 x 10^5 per µl respectively. The mean platelet count of diseased animal was decreased, suggestive of thrombocytopenia. Only 50 per cent animals had shown a marked reduction in the platelet count indicating thrombocytopenia.

4.3.4 Packed Cell Volume (PCV)

The mean packed cell volume of the diseased and control were 32.37 ± 3.66 per cent and 44.93 ± 2.45 per cent respectively. The statistical analysis showed a significant difference was there in these groups.

4.3.5 Haemoglobin

The mean Haemoglobin of the diseased and control were 11.05 ± 1.34 g/dl and 14.15 ± 0.37 g/dl respectively. Difference between the diseased and control groups were statistically significant.

Table 6. Mean haematological values of control and diseased animals

Parameter	Mean ± SE Control	Mean ± SE Diseased
RBC (x 10^6 / µl)	6.89 ± 0.11	4.92 ± 0.67*
WBC (x 10^3 / µl)	10.15 ± 0.61	37.13 ± 8.67*
Neutrophil (per cent)	71.67 ± 1.52	79.83 ± 2.77*
Lymphocyte (per cent)	25.33 ± 1.31	16.67 ± 2.24*
Monocyte (per cent)	2 ± 0.26	1.25 ± 0.57 ns
Eosinophil (per cent)	1 ± 0.36	1.42 ± 0.26 ns
Platelet (x 10^5 / µl)	3.67 ± 0.11	1.91 ± 0.34*
Haemoglobin (g/dl)	14.15 ± 0.37	11.05 ± 1.34*
PCV (per cent)	44.93 ± 2.45	32.37 ± 3.66*

* - Significant

ns - Non significant

Table 7. Haematological values of diseased animals

SI No.	Case No.	RBC ($x10^6/\mu l$)	WBC ($x10^3/\mu l$)	Neutrophil (%)	Lymphocyte (%)	Monocyte (%)	Eosinophil (%)	Platelet ($x10^5/\mu l$)	Hb (g/dl)	PCV (%)
1	4800	4.92	30900	75	20	3	2	115000	12.3	31.3
2	6206	6.47	14400	76	22	0	2	86000	12.8	36.6
3	962	6.15	45800	87	8	5	0	120000	14.3	37.7
4	8773	3.3	19000	82	18	0	0	218000	9.6	32
5	6455	5.2	10100	92	5	1	2	90000	9	26
6	10336	2.59	21700	70	21	5	2	398000	6.11	18
7	7479	4	19600	86	12	0	2	198000	9.68	35
8	951	7.93	40900	70	28	1	1	200000	15.9	47.4
9	8429	1.3	104000	88	12	0	0	88000	4	12
10	361	1.83	90400	86	12	0	2	397000	4.7	15.1
11	8724	7.67	20100	86	12	0	2	295000	17.8	48.7
12	4735	7.7	28600	60	30	0	2	92000	16.4	48.6

4.4 Biochemical analysis

The mean biochemical values and of control and diseased animals are presented in Table 8 and the results of biochemical parameters of diseased animals are given in Table 9.

4.4.1 Serum Creatinine

The mean serum creatinine was apparently higher in diseased dogs and it was 6.07 ± 1.87 mg/dl and 1.15 ± 0.07 mg/dl in control group of dogs, which was statistically significant. The increased mean serum creatinine showed the animals were azotemic. A high value of serum creatinine (above 2.5 mg /dl) was shown by 58.33 per cent animals (7 out of 12).

4.4.2 Serum Alanine amino transferase (ALT)

The mean serum alanine amino transferase values in diseased and control dogs were 69.9 ± 11.66 U/L and 19.92 ± 1.42 U/L respectively. The increase in the mean alanine amino transferase was statistically significant in diseased dogs.

4.4.3 Total Protein, Albumin and Globulin

4.4.3.1 Serum Total Protein

The mean serum total protein was 6.51 ± 0.33 g/dl and 7.1 ± 0.13 g/dl respectively in the diseased and control groups. The mean count in diseased group was statistically significant.

4.4.3.2 Serum Albumin

The difference between mean serum albumin between diseased and control group was significant, with mean values at 2.38 ± 0.17 g/dl and 3.7 ± 0.17 g/dl respectively.

4.4.3.3 Serum Globulin

The mean serum globulin of the diseased and control were 4.13 ± 0.28 g/dl and 3.4 ± 0.12 g/dl respectively. The statistical analysis showed a significant difference was there in these groups.

4.4.4 Albumin – Globulin ratio (A:G)

The mean Albumin – globulin ratio of the diseased and control groups were 0.61 ± 0.06 and 1.12 ± 0.08 respectively. Difference between the diseased and control were statistically significant.

4.4.5 Serum Total Bilirubin

The difference between mean serum total bilirubin between diseased and control group was significant, with mean values at 4.25 ± 1.66 mg/dl and 0.20 ± 0.10 mg/dl respectively, suggestive of hyperbilirubinemia. Fifty per cent (6 out of 12) animals showed a high serum total bilirubin of above 4 mg/dl.

4.4.5.1 Direct Bilirubin

The difference between mean serum direct bilirubin between diseased and control group was significant, with mean values at 2.94 ± 1.22 mg/dl and 0.06 ± 0.12 mg/dl respectively.

4.4.5.2 Indirect Bilirubin

The mean serum indirect bilirubin of the diseased and control were 1.32 ± 0.50 mg/dl and 0.20 ± 0.18 mg/dl respectively. The statistical analysis showed a significant difference was there in these groups.

4.5 Ultrasonography

Abdominal ultrasonographic findings are summarized in Table 10.

Table 8. Mean biochemical values of control and diseased animals

Parameter	Mean ± SE Control	Mean ± SE Diseased
Creatinine (mg/dl)	1.15 ± 0.07	6.07 ± 1.87*
ALT (U/L)	19.92 ± 1.42	69.9 ± 11.66*
Total Protein (g/dl)	7.1 ± 0.13	6.51 ± 0.33*
Albumin (g/dl)	3.7 ± 0.17	2.38 ± 0.17*
Globulin (g/dl)	3.4 ± 0.12	4.13 ± 0.28*
A/G ratio	1.12 ± 0.08	0.61 ± 0.06*
Total Bilirubin (mg/dl)	0.20 ± 0.10	4.25 ± 1.66*
Direct Bilirubin (mg/dl)	0.06 ± 0.12	2.94 ± 1.22*
Indirect Bilirubin (mg/dl)	0.20 ± 0.18	1.32 ± 0.50*

* - Significant

Table 9. Biochemical values of diseased animals

SI No.	Case No.	Creatinine (mg/dl)	ALT (U/L)	Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G	Bilirubin (mg/dl)	Bilirubin (Direct) (mg/dl)	Bilirubin (Indirect) (mg/dl)
1	4800	1.3	29	6.8	1.6	5.2	0.31	0.1	0.1	0
2	6206	0.7	107	5.7	1.4	4.3	0.33	4.7	4.2	0.5
3	962	2.6	149	4.5	2.3	2.2	1.05	10.5	8.3	2.2
4	8773	19.9	49	6.1	2.5	3.6	0.69	0.3	0.1	0.2
5	6455	12.4	71	7.6	2.4	5.2	0.46	0.4	0	0.4
6	10336	4.9	57	6.6	3	3.6	0.83	0.3	0.2	0.1
7	7479	6.8	64	6.9	3	3.9	0.77	17	12.2	4.8
8	951	15.7		9.2	3.3	5.9	0.56	44.24	27.9	16.34
9	8429	0.8	45	5.8	2.4	3.4	0.71	6.3	3.7	2.6
10	361	2.2	495	6.2	1.8	4.4	0.41	6.5	3.2	3.3
11	8724	4.9	19	6.7	2.8	3.9	0.72	0.3	0.1	0.2
12	4735	0.6	109	6	2	4	0.50	0.4	0.2	0.2

On abdominal ultrasonography of leptospirosis dogs (11 cases), cortico medullary junction of kidney was not clear in four cases (36.36 per cent). Kidney cortex was hypo echoic in two cases and hyper echoic in one case. No change in echogenicity of kidney noted in three cases (27.27 per cent) (Plate 6).

Ultrasonography of liver revealed hypo echogenicity in three cases (27.27 per cent) and slight enlargement of liver noted in 2 cases. But no abnormality in echogenicity and architecture noted in 5 cases (45.45 per cent) (Plate 7).

Splenomegaly noted in two cases (18.18 per cent).

4.6 Urinalysis

Urinalysis was done in 12 cases and summarized in Table 11.

Specific gravity was in a range of 1.01 to 1.03. Proteinuria was observed in most cases (75 per cent). Presence of blood in urine observed in 25 per cent cases. Glucosuria was there in 25 per cent cases. Bile pigment was detected in 41.67 per cent dogs. Bile salt was absent in all the samples tested.

4.7 Treatment

Positive cases were divided into two groups of six animals each. One group was treated with Benzyl penicillin and other group treated with Amoxicillin - Clavulanic acid. Supportive therapy was also maintained along with antibiotic treatment. But only three animals (25 per cent) survived after treatment and rest of the animals died before completing the course of therapy.

Out of the three animals survived, two animals (Case no. 4800 and 8724) were treated with Amoxicillin - Clavulanic acid and one animal (Case no. 10336) with Benzyl penicillin.

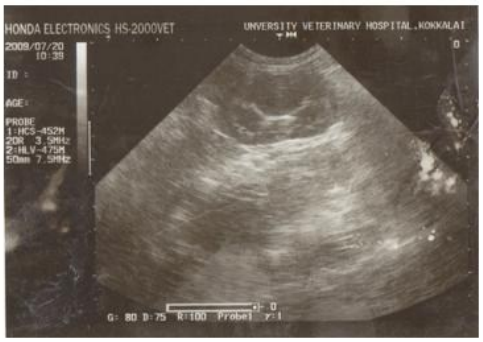


Plate 6 – Ultrasonogram
Kidney - Cortico medullary distinction not clear

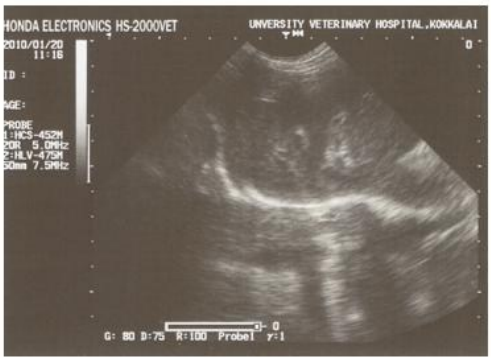


Plate 7 - Ultrasonogram
Liver - Hypo echoic and Hepatomegaly

Table 10. Ultrasonographic observations

SI No.	Case No.	Kidney	Liver	Spleen
1	361	NAD	Discrete hyper echoic foci	NAD
2	951	Cortico-medullary junction not clear, Medulla - enlarged and fluid filled	Slight enlargement of liver with mixed echogenicity	NAD
3	962	Cortex – hyper echoic	Discrete hypo echoic areas noted	NAD
4	4735	Cortico-medullary junction not clear	NAD	NAD
5	4800	Right kidney enlargement, Cortex – hyper echoic	Blood vessels are congested and hypo echoic liver	NAD
6	6206	Slight enlargement with hypo echoic cortex.	NAD	Splenomegaly with congestion of splenic vessels
7	6455	Cortico-medullary junction not clear	NAD	NAD
8	7479	Left kidney cortex anechoic and not distinct	Certain anechoic areas noted	NAD
9	8724	NAD	NAD	Splenomegaly
10	8773	Cortico-medullary junction not clear	NAD	NAD
11	10366	NAD	Hepatomegaly, Hypo echoic areas observed with interrupted hyper echoic area	Congestion of splenic vessels

NAD – No abnormality detected

Table 11. Urinalysis

S.I. No.	Case no.	Specific gravity	Blood	Glucose	Protein	Bile pigment	Bile salt
1	4800	1.016	+	-	+	-	-
2	6206	1.01	-	-	-	+	-
3	962		-	-	+	+	-
4	8773	1.015	-	-	+	-	-
5	6455	1.03	-	-	-	-	-
6	10336	1.01	-	-	+	-	-
7	7479	1.014	-	+	+	+	-
8	951	1.03	-	+	+	+	-
9	8429	1.01	-	+	+	+	-
10	361	1.022	-	-	+	-	-
11	8724	1.015	+	-	+	-	-
12	4735	1.004	+	-	-	-	-

Discussion

5. DISCUSSION

The present study was carried out in clinically suspected cases of leptospirosis in dogs. These animals were screened for leptospirosis with dark field microscopic examination, microscopic agglutination test and PCR in appropriate clinical samples. The positive cases were evaluated in detail and treatment done. Haematological and biochemical analysis, ultrasound evaluation, and urinalysis were carried out.

5.1 Examination of Leptospires in suspected cases

5.1.1 Dark Field Microscopy

Demonstration of leptospires in blood and urine is one of the confirmatory tests in the diagnosis of leptospirosis. Present study involved screening clinical samples of suspected cases with DFM, but failed to detect any organism in blood or urine samples. It was in agreement with report of many researchers (Kalin *et al*., 1999 and Magajevski *et al*., 2005), who could not observe leptospires in clinical samples of dogs, even with positive antibody titre under DFM. But *Leptospira* was demonstrated in the blood and urine with DFM (Elaiyaraja, 2003 and Zaragoza *et al.,* 2003).

In the present study, no samples revealed leptospiral organism in wet mount preparations. DFM could detect leptospires only when large numbers of viable leptospires were present in the clinical samples. Also, absence of leptospires in the urine under DFM might be due to the intermittent shedding of organism or its poor survivability in acidic urine (Birnbaum *et al*. 1998). Moreover, leptospiruria is mainly seen in convalescent and recovering dogs, that during the acute stage.

Leptospiral movement in wet mount preparations might be confused with other cellular fibrils or fibrin strands (Greene *et al.*, 2006). So direct visualization of leptospires in fresh specimens by DFM would be very difficult

and could not be considered as a reliable diagnostic procedure (Vijayachari *et al*., 2001).

5.1.2 MAT

MAT is considered as the gold standard for the diagnosis of leptospirosis in dogs. Detection of serum antibodies using MAT is the most commonly used diagnostic method for leptospirosis in dogs (Songer and Thiermann, 1988).

Out of the 20 sera samples tested 12 (60 per cent) were positive for antibodies to *Leptospira* serovars. But Indu (1997) reported a seropositivity of 32.66 per cent where as Nair (2004) reported 12.38 per cent for leptospires among dogs in and around Thrissur district. This result indicated an increase in occurrence of leptospirosis among dogs in Thrissur. This might be due to increased contact between dogs and wildlife reservoirs in expanding suburban environments.

The present study showed the highest prevalence of antibody to *L. autumnalis* and *L. australis* (75 per cent each). But this finding was in contrary to result of other studies (Indu, 1997 and Soman, 2004), that showed *Leptospira pomona* as a predominant serovar among dogs in and around Thrissur. The predominant titres were *L. autumnalis and L. australis* in 75 per cent dogs, which suggests that these serovars are the most common etiologic agent of leptospirosis in dogs in Thrissur. These data indicated that there had been a change from the traditional serovars that typically caused disease in dogs. The emergence of serovar autumnalis and australis, other than vaccinal serovars in dogs probably due to epizootic changes in canine leptospirosis.

Vaccination of dogs with the currently available bivalent and quadrivalent vaccine against *L. icterohaemorrhagiae*, *L. canicola, L. pomona* and *L. grippotyphosa* being serovar specific offers no protection against infection with other serovars. So development of a multivalent vaccine with serovars of higher

occurrence that results in immunity to these serovars is also necessary (Adin and Cowgill, 2000).

5.1.3 PCR

Leptospires could be identified in biomaterials by molecular methods (Dhannia, 2005). For early diagnosis of leptospirosis, PCR would be a promising approach and it would be more sensitive and rapid than culturing (Bal *et al.,* 1994 and Lilenbaum *et al*., 2009).

In the present study, only two animals found positive by PCR, even though most of the remaining animals were also seroreactive. Although PCR analysis of clinical materials was more sensitive than culturing, a disappointing major proportion are missed by PCR, probably because the number of leptospires in these samples were too small (Bal *et al.,* 1994) or absent to be detected by PCR. Also, shedding of organism would be intermittent in case of urine (Birnbaum *et al.,* 1998) and so repeated collection of sample may be required for detection of organism in urine.

5.2 History and Clinical manifestation of leptospirosis in dogs

5.2.1 Sex

Here, most of the affected were females. Among positive cases 58.33 per cent were females and 41.67 per cent were males. The increased incidence of cases in female dogs might be due to the increase in number of female population. This was in contrary to report of predominance of males in affected cases (Adin and Cowgill, 2000 and Claus *et al*., 2008).

5.2.2 Age

The affected animals were from an age group of one to six years with an average of 3.3 years. It was in agreement with a previous study by Geisen *et al*. (2007), where the affected dogs were predominantly middle aged dogs. Middle

aged dogs might be more active outside their normal home environment than young or old dogs, increasing potential exposure to *Leptospira* serovars (Ward *et al*., 2004).

5.2.3 Breed

In the present study the affected dogs were predominantly large breed dogs and more incidences noted on Labrador Retrievers (33.3 per cent). Similar observations had also been reported by Adin and Cowgill (2000), who found more affected cases were from Labrador Retrievers and German Shepherd dogs.

5.2.4 Season

In the present study, the cases presented mostly during rainy season of the year (July to November) though leptospirosis can occur at any time of the year. A strong association between periods of high rainfall and leptospirosis had been documented. In the monsoon the contamination of surface water got increased and more outbreaks were happened in this season (Greene *et al*., 2006 and Kuriakose *et al*., 2008).

5.2.5 Clinical signs

Clinical observations in canine leptospirosis were non specific in that they reflected multi systemic organ failure rather than a specific disease (Miller *et al*., 2007). In the present study, most predominant clinical manifestations of dogs with leptospirosis included inappetence, vomiting and jaundice as reported previously (Schreiber *et al*., 2005; Geisen *et al.,* 2007; Miller *et al*., 2007 and Minke *et al*., 2009). This result showed involvement of the various organ systems of the body and which would be a multi organ disease pattern (Miller *et al*., 2007 and Minke *et al*., 2009).

Among the positive cases jaundice was one of the most predominant signs and so leptospirosis should be considered as a differential diagnosis for animals presenting with liver disease even in the absence of renal failure (Boutilier *et al*.,

2003). Second most predominant signs were haematuria (25 per cent), and melaena (25 per cent). Consistent with previous case reports and results of retrospective studies several workers had reported affected dogs with microscopic haematuria, red to brownish coloured urine and bloody diarrhoea (Birnbaum *et al*., 1998; Geisen *et al*., 2007 and Minke *et al.*, 2009).

Most of the infected dogs were not in febrile stage and 16.67 per cent dogs were with slight reduction in temperature. This might be due to the affected serovar, which determined increase or decrease in temperature of the affected animal (Klaasen *et al*., 2003).

5.3 Haematology

Das *et al*. (2004) suggested that haemato-biochemical analysis should be conducted along with other tests for the confirmatory diagnosis of leptospirosis.

5.3.1 Total Leucocyte Count and Differential Leucocyte Counts

Most of the affected animals exhibited a profound leucocytosis with a prominent neutrophilia and lymphopenia. This might be due to the stimulation of neutrophil adherence and activation which might be involved in inflammatory and coagulatory abnormalities (Chandrasekaran *et al.*, 2009).

5.3.1a Total leucocyte count

In the present study, there was marked increase in total leucocyte count showed by the most of the diseased animals**.** Similar observations had also been reported in dogs with leptospirosis by several workers (Birnbaum *et al*., 1998; Boutilier *et al*., 2003; Zaragoza *et al*., 2003; Schreiber *et al*., 2005 and Geisen *et al.,* 2007).

5.3.1b Neutrophil count

The neutrophil count between diseased and control group was significant and about more than 50 per cent animals showed neutrophilia. Leucocytosis with

neutrophilia in leptospirosis in dogs was also recorded by Adin and Cowgill (2000) and Geisen *et al.* (2007).

5.3.1c Lymphocyte count

The slight reduction in mean lymphocyte counts in diseased animals was significant and it corroborated the findings of Jana *et al.* (2004) and Chandrasekaran *et al.* (2009).

5.3.1d Eosinophil count

There was no significant difference observed on mean eosinophil count of the diseased and control group. This finding contradicts results of a previous study in dogs of leptospirosis, in which a decrease in eosinophil counts noted (Jana *et al*., 2004).

5.3.1e Monocyte count

The monocyte count was found normal for the diseased animals and which was in contrary to the findings of Klaasen *et al*. (2003) and Jana *et al*. (2004).

5.3.2 Total Erythrocyte Count

More than 50 per cent of animals showed a marked reduction in erythrocyte count suggestive of anaemia. This finding was supported by the prior findings of Birnbaum *et al*. (1998) who observed a mild non regenerative anaemia in 33 per cent of dogs and Geisen *et al.* (2007) found anaemia in 45 per cent dogs. Decrease in RBC and haemoglobin concentration leptospirosis might be due to the haemorrhage and blood loss in the disease process (Jana *et al*., 2004).

5.3.3 Platelet Count

In the present study 50 per cent animals had shown a marked reduction in the platelet count indicating thrombocytopenia which was in agreement with findings of Adin and Cowgill (2000) who found 55 per cent dogs had

thrombocytopenia in affected cases. Similar observations had also been reported by several other workers (Schreiber *et al*., 2005; Geisen *et al.,* 2007 and Claus *et al.,* 2008). This finding contradicts results of a previous study of leptospirosis in dogs, in which thrombocytopenia was not observed (Boutilier *et al*., 2003).

The accelerated platelet clearance by the platelet aggregation and Kupffer cells phagocytosis might be the potential causes of thrombocytopenia in severe leptospirosis rather than diminished production (Yang *et al*., 2006).

5.3.4 Packed Cell Volume (PCV) and Haemoglobin

The mean packed cell volume and mean haemoglobin of the diseased and control showed a statistically significant difference between both groups. This was in agreement with previous reports (Zaragoza *et al*., 2003; Jana *et al*., 2004; Claus *et al.,* 2008 and Chandrasekaran *et al.,* 2009). But Jamshidi *et al.* (2008) reported a PCV of 60 per cent due to haemoconcentration in a clinical case of leptospirosis in dog.

5.4 Biochemical analysis

5.4.1 Serum Creatinine

A significant increase in the mean serum creatinine was observed in positive dogs. This finding was supported by the prior finding of several researchers (Boutilier *et al.,* 2003; Geisen *et al.,* 2007; Chandrasekaran *et al*., 2009 and Minke *et al.*, 2009).

In the present study, more than 50 per cent animals showed a high value of serum creatinine. The increased mean serum creatinine indicated that the animals were azotemic during the initial presentation (Adin and Cowgill, 2000).

Renal failure was the typical response to leptospiral infection in an unadapted host such as the dog (Birnbaum *et al*., 1998 and Golstein *et al*., 2006). Following localisation in the renal tubules, it was proposed that leptospiral toxins

cause necrosis of adjacent tubular cells with resultant nephrosis and subsequent renal failure (Miller *et al*., 2007) resulting in an increased level of serum creatinine.

5.4.2 Serum Alanine amino transferase (ALT)

In the present study the elevated mean serum alanine amino transferase activity was statistically significant in diseased dogs. Similar observations had also been reported by several researchers (Boutilier *et al.,* 2003; Jana *et al*., 2004; Miller *et al*., 2007 and Claus *et al.,* 2008).

The minimally elevated ALT values in the present study indicated that there was limited hepatocellular necrosis (Miller *et al*., 2007).

5.4.3 Total Protein, Albumin and Globulin

The mean serum total protein, mean serum albumin and mean serum globulin between diseased and control group were significant. There was a slight reduction in the mean total protein of the diseased animals which was in agreement with Claus *et al.* (2008). But in contrary an increased serum total protein was reported by Jana *et al*., 2004).

Mean serum albumin was slightly reduced in positive animals. This finding was similar to results of other studies (Zaragoza *et al*., 2003; Jana *et al*., 2004 and Claus *et al.,* 2008). The mean serum globulin of the diseased animals was slightly increased. It might be due the production of more antibodies towards the infecting organisms.

5.4.4 Albumin – Globulin ratio (A:G)

The increase in the globulin and decrease in albumin content made a reduction in A:G ratio of diseased animals which was statistically significant.

5.4.5 Serum Total Bilirubin, Direct and Indirect Bilirubin

Mean serum total bilirubin, mean serum direct and indirect bilirubin of the diseased animals was statistically significant. There was an increase in the mean values noted in affected group.

In the present study 50 per cent animals were hyperbilirubinemic and this was in correlation with the prior finding of hyperbilirubinemia in 22 per cent dogs by Adin and Cowgill (2000) and 79 per cent dogs by Geisen *et al.* (2007). Similar observations had also been reported by several other workers (Schreiber *et al*., 2005; Miller *et al*., 2007; Chandrasekaran *et al*., 2009 and Minke *et al.*, 2009).

Hepatic cholestasis and hepatic pathological changes as evidenced by increased bilirubin level was marked in the affected groups. Icterus appears to be the result of hepatic cell damage due to obstruction of canaliculi.

5.5 Ultrasonography

Abdominal ultrasound of kidney would be useful for evaluating early morphological changes (Prathapan, 2009). In acute or chronic stage, the localization of organism in the kidney tubules would create morphological abnormalities in the surface of kidney and liver, which could be evaluated by ultrasonography.

In the present study, abdominal ultrasonography of dogs with leptospirosis had done and found cortico medullary junction of kidney was not clear in most of the cases, which was in agreement with the report of Prathapan (2009). Kidney cortex was hypo echoic in two cases and in one case hyper echoic kidney cortex was noticed. This observation was in agreement with the findings reported earlier by Birnbaum *et al*. (1998) and Forrest *et al*. (1998). No change in renal architecture and echogenicity was noted in three cases (27.27 per cent) which

were in accordance with the findings of Prescott *et al.* (1991) and Adin and Cowgill (2000).

Although the results of the abdominal ultrasound tests were non-specific, they revealed abnormalities in the echogenicity or architecture of the kidneys in four out of the eleven cases. Therefore, this test had the potential to play a role in earlier diagnosis of canine leptospirosis (Forrest *et al*., 1998).

5.6 Urinalysis

5.6.1 Specific Gravity

In the present study urinalysis of positive cases revealed most dogs were isosthenuric (Adin and Cowgill, 2000; Claus *et al*., 2008 and Okewole and Ayoola, 2009) and urine specific gravity was between 1.004 to 1.016 in 72.72 per cent animals.

Urine concentrating ability was retained by 27.28 percent dogs with values ranging from 1.022 to 1.03 in the present study, which was in agreement with findings of Okewole and Ayoola (2009) they found only 9.6 per cent dogs retained their urine concentrating ability. Most of these animals had concurrent azotemia and the low urine specific gravity was consistent with renal failure. Similar observations had also been reported by Birnbaum *et a*l. (1998) and Kalin *et al*. (1999).

5.6.2 Protein

In the present study proteinuria was observed in 75 per cent cases. It substantiated the findings of Geisen *et al*. (2007) who reported 71 per cent of leptospirosis dogs showed proteinuria. Several other workers also reported proteinuria (Prescott *et al*., 1991; Claus *et al*., 2008 and Jamshidi *et al.*, 2008).

Acute tubular damage could explain proteinuria in many of these dogs on the basis of additional findings such as glycosuria (Goldstein *et al*., 2006)

5.6.3 Blood

Adin and Cowgill (2000) reported 17 per cent dogs had microscopic evidence of haematuria in leptospirosis cases in dogs. Haematuria was observed in 25 per cent cases each in the present study. This observation was in agreement with the findings reported earlier (Prescott *et al*., 1991; Birnbaum *et al*., 1998; and Kalin *et al*., 1999).

In the present study, haemoglobinuria also observed in 25 per cent cases. This study was supported by the prior finding of Claus *et al*. (2008) who detected haemoglobinuria in a dog with leptospirosis.

5.6.4 Bile pigment and Bile salt

In this study 41.67 dogs showed bile pigments in urine. It was in agreement with findings of Jamshidi *et al.* (2008) and Okewole and Ayoola (2009).

Bilirubin levels might be increased in serum and urine, and the magnitude was usually proportional to the degree of liver impairment. Marked bilirubinuria usually precedes hyperbilirubinemia (Greene *et al*., 2006). Bile salt was absent in all the samples tested.

5.6.5 Glucose

On urinalysis it was found that 25 per cent affected cases showed glucosuria which was in agreement with Adin and Cowgill (2000) they detected glucosuria in 26 per cent dogs. Similar findings in leptospiral infection were observed by Birnbaum *et al*. (1998); Kalin *et al.* (1999) and Claus *et al*. (2008). Glucosuria without hyperglycemia, which occurred in leptospirosis dogs, suggested a renal tubular dysfunction (Greenlee *et al*., 2004).

5.7 Treatment response

The prognosis of leptospirosis in dogs is fair to poor, depending on the clinical state of the patient at initial presentation and on the causative leptospiral serovar (Claus *et al.,* 2008).

Survival rate was only 25 per cent in the present study. This finding was in agreement with previous reports by Miller *et al*. (2007) who recorded a mortality rate of more than 50 per cent in dogs with leptospirosis in a retrospective study. In the present study, a high mortality rate noted in positive cases that might be due to infection with non host adapted serovars, which resulted in acute form of leptospirosis and death (Heath and Johnson, 1994).

Penicillin and its derivative is the antibiotic of choice for leptospirosis and it minimizes organ damage and quickly clears the leptospiraemic phase (Greene *et al*., 2006). But if the diagnosis and treatment is delayed, prognosis would be grave. In the present study, since the number of animals survived in each group was very less, comparison of efficiency of antibiotic therapy was not possible. Two animals survived in Amoxicillin - Clavulanic acid group, but only one in Benzyl penicillin group.

Haemodialysis had been shown to improve the prognosis of dogs with leptospirosis and severe azotemia (Adin and Cowgill, 2000). Treating every patient with clinical signs and laboratory abnormalities of acute renal failure without a known cause using penicillin derivatives until specific testing confirms or rules out leptospirosis helps to give a good prognosis (Claus *et al.*, 2008).

Summary

6. SUMMARY

Leptospirosis is a zoonosis of world wide occurrence and dogs are considered to be convalescent carriers for many pathogenic leptospires. The symptoms of leptospirosis are non-specific, which resemble a wide range of bacterial and viral diseases. So a prompt diagnosis of leptospirosis is of paramount importance.

Dogs presented in Veterinary Hospitals Mannuthy and Kokkalai with clinical signs suggestive of leptospirosis were considered in the present study. About 20 suspected cases were taken and studied in detail. The blood and urine samples of these animals were collected and screened by dark field examination. PCR was also done to detect the pathogenic DNA of the leptospires in these samples. The sera collected and MAT was performed to detect agglutinating antibodies to different leptospira serovars.

DFM examination did not reveal motile organisms in any of the blood or urine samples tested. PCR could identify leptospiral DNA in one blood and one urine sample.

On MAT, 12 sera were found positive for leptospiral agglutinating antibodies. Predominant seroreactions were to *L. autumnalis* and *L. australis* in 75 per cent cases. Among seropositive dogs 50 per cent cases reacted at a higher titre of 1:400 or more, and remaining cases reacted to at low titre of 1:100. One serum sample (case no. 7479) had shown a high titre of 1:3200 against *L. icterohaemorrhagiae, L. hebdomadis* and *L. javanica*. The predominant titres were *L. autumnalis and L. australis* in 75 per cent dogs, which suggests that these serovars are the most common etiologic agent of leptospirosis in dogs in Thrissur.

On analysing clinical manifestation of cases, animals were presented with inappetence, anorexia, vomiting, jaundice, haematuria, haemoglobinuria and melaena. Temperature was within the normal range in majority of cases. The

occurrence was found to be more in female dogs. Middle aged dogs were found to be more affected. In the present study the occurrence was higher during the monsoon season (July to November).

Haemato-biochemical changes may provide an effective indicator in the prognosis of clinical canine leptospirosis. Haemogram of all the positive cases revealed most of the haematological parameters were significant except mean monocyte and eosinophil count.

A marked reduction in erythrocyte count observed in 58.33 per cent animals suggestive of anaemia. There was marked increase in total leucocyte count showed by the most of the diseased animals. About 91.67 per cent animals showed a leucocyte count more than 17,000 per µl. Neutrophilia, lymphopenia, a decrease in mean PCV and haemoglobin concentration also noted in seropositive animals. Only 50 per cent animals had shown a marked reduction in the platelet count indicating thrombocytopenia.

The mean biochemical values of diseased animals were detected. The mean serum creatinine was apparently higher in diseased dogs, which was statistically significant. The increased mean serum creatinine showed the animals were azotemic. A high value of serum creatinine above 2.5 mg/dl was showed by 58.33 per cent animals. The mean ALT, total protein, albumin, globulin and total bilirubin were statistically significant in diseased dogs. A high serum total bilirubin of above 4 mg/dl was shown by 50 per cent animals.

On abdominal ultrasonography of dogs with leptospirosis, cortico medullary junction of kidney was not clear in 36.36 per cent cases. Kidney cortex was hypo echoic in two cases and hyper echoic in one case. No change in echogenicity of kidney noted in 27.27 per cent cases. Ultrasonography of liver revealed no abnormality in echogenicity and architecture in 45.45 per cent cases.

Urinalysis of positive cases had done. Specific gravity was in a range of 1.01 to 1.03. Proteinuria was observed in 75 per cent cases. Presence of blood

observed in 25 per cent cases. Glucosuria was there in 25 per cent cases. Bile pigment was detected in 41.67 per cent dogs. Bile salt was absent in all the samples tested.

Positive cases were divided into two groups. One group with six animals was treated with Benzyl penicillin and other group containing six animals treated with Amoxicillin - Clavulanic acid. But only 25 per cent animals were survived after treatment and rest of the animals were died before completing the course of therapy.

A change in the epidemiology of leptospirosis in dogs, with prevalence of agglutinins to *L. autumnalis* and *L. australis* were noted in the present study. These data indicated that there had been a change from the traditional serovars that typically caused disease in dogs in Thrissur. Clinical manifestations were indicative of a multisystemic involvement. Among the positive cases jaundice was one of the most predominant signs and so leptospirosis should be considered as a differential diagnosis for animals presenting with liver disease even in the absence of renal failure. Abdominal ultrasound had the potential role to play in the earlier diagnosis of canine leptospirosis. Most of these animals had concurrent azotemia and the low urine specific gravity was consistent with renal failure. A very less survival rate was noted in the present study.

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CLINICO-THERAPEUTIC STUDIES ON LEPTOSPIROSIS IN DOGS

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ABSTRACT

Clinico-therapeutic studies of leptospirosis in dogs presented in the University Veterinary Hospitals, Mannuthy and Kokkalai were conducted. About 20 suspected cases were taken and screened for leptospirosis by DFM, PCR and MAT.

Leptospiral organism could not be observed by DFM examination from any of the blood or urine samples tested. But PCR could amplify pathogenic leptospiral DNA in one blood and one urine sample. On MAT, 12 sera were found positive for leptospiral agglutinating antibodies. The predominant seroreactions were to *L. autumnalis* and *L. australis* in 75 per cent cases.

Clinical manifestations were indicative of a multisystemic involvement. Most of the animals were presented with inappetence, anorexia, vomiting, jaundice, haematuria, haemoglobinuria, melaena and normal body temperature. More cases reported in female dogs. Middle aged dogs were affected more. In the present study the incidence was more during the monsoon season.

On haematological analysis, most of the haematological parameters were significant in all the positive cases except mean monocyte and eosinophil count. A marked reduction in erythrocyte count observed in 58.33 per cent animals suggestive of anaemia. There was marked increase in total leucocyte count showed by the most of the diseased animals. Neutrophilia, lymphopenia, a decrease in mean PCV and haemoglobin concentration also noted in seropositive animals. Thrombocytopenia was observed in 50 per cent animals.

In positive cases the mean serum creatinine was apparently higher. The increased mean serum creatinine showed the animals were azotemic. The mean ALT, total protein, albumin, globulin and total bilirubin were significantly higher in diseased dogs.

On abdominal ultrasonography of dogs with leptospirosis, cortico medullary junction of kidney was not clear in 36.36 per cent cases. No change in echogenicity of kidney noted in 27.27 per cent cases. Ultrasonography of liver revealed no abnormality in echogenicity and architecture in 45.45 per cent cases.

On urinalysis, specific gravity was in a range of 1.01 to 1.03. Proteinuria was observed in 75 per cent cases. Presence of blood observed in 25 per cent cases. Glucosuria was there in 25 per cent cases. Bile pigment was detected in 41.67 per cent dogs. Bile salt was absent in all the samples tested.

Positive cases were divided into two groups. One group with six animals was treated with Benzyl penicillin and other group containing six animals treated with Amoxicillin - Clavulanic acid. But only 25 per cent animals were survived after treatment and rest of the animals were died before completing the course of therapy.