SEROVARS AND RENAL PATHOLOGY OF LEPTOSPIROSIS IN DOGS, CATTLE AND WILD RATS, AND ITS PATHOGENESIS IN A GUINEA PIG MODEL

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CERTIFICATION

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DEDICATION

This work is dedicated to the great God of heaven, the giver of life and understanding. As Richard Baxter rightly put it when he said "Nothing can be rightly known, if God be not known, nor is any study well managed, nor to any great purpose, if God is not studied. We know little of the creature, till we know it as it stands related to the creator: single letters, and syllables uncomposed, are no better than nonsense and he who overlooked Him who is the Alpha and Omega, the beginning and the ending, and sees Him not in all, who is the All of all do not see at all. Such creatures are broken syllables and they signify nothing".

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ABSTRACT

In recent years, mortality due to leptospirosis in dogs has been of major concern in Southwestern Nigeria, but the prevalent serovars, pathology and pathogenesis have not been fully documented. The purpose of this study was to investigate the disease in the dog and reservoir hosts (cattle and wild rats), and its pathogenesis and pathology in a guinea pig model.

Between 2003 and 2010, 44 fresh and 60 formalin-fixed kidneys of tentatively diagnosed cases of canine leptospirosis obtained at necropsy from the two Veterinary Teaching Hospitals in Southwestern Nigeria, fresh kidneys of 108 asymptomatic cattle and 105 wild rats obtained from abattoirs were investigated histopathologically and for the presence of leptospiral organisms using Ellinghausen-McCullough-Johnson–Harris (EMJH) medium, microscopic agglutination test with monoclonal antibodies (MAT-Ab), polymerase chain reaction (PCR), Warthin Starry silver stain (WSss), and immunohistochemistry (IH). The chronicity of the canine infection was determined by renal histopathology. Forty guinea pigs were experimentally infected with 10^7 *Leptospira interrogans* serovar icterohaemorrhagiae, while 20 served as controls. The clinico-pathological changes, pathogenesis and pathology were investigated by sacrificing at 12, 24, 72, 96, 120 and 168 hrs post infection (p.i.). Data was analysed using descriptive statistics.

Interstitial nephritis and tubular nephrosis were the most common renal lesions in dogs (88.4% and 76.7%), cattle (77.8% and 74.1%) and wild rats (62.9% and 67.6%), respectively. Leptospires were isolated from 84.1% dogs, 82.4% cattle and 68.9% wild rats. Serovars identified in dogs, cattle and wild rats were icterohaemorrhagiae (29.7%, 9.7%, 33.0%), pomona (18.5%, 3.2%, 23.8%), bratislava (11.1%, 22.6%, 4.8%), hardjo (0%, 29.0%, 0%), canicola (14.8%, 6.5%, 14.3%) and grippotyphosa (14.8%, 9.7%, 14.3%); unidentified isolates were 11.1%, 19.4%, 9.5% respectively. The virulence gene (285bp) was confirmed in

31.3% of canine, 81.8% of wild rat and 61.9% of bovine kidneys. The IH and WSss showed that in acute canine infections, leptospires were present in different renal tissues, but were only found attached to tubular epithelium in the more chronic infections. The guinea pig infection was characterized by anorexia, dullness, slight icterus, normocytic normochromic anemia, and thrombocytopenia. The detection of leptospires in different organs was time-dependent, but persisted only in the kidney after 120 hrs p.i. In the pancreas, the organism could only be detected by cultural isolation and PCR (12-72 hrs p.i.). Renal tubular necrosis and interstitial nephritis, hepatic necrosis and cord dissociation, pulmonary haemorrhages, acute pancreatitis, adrenal vacuolar degeneration, non-suppurative myocarditis and encephalitis were observed. Immunohistochemistry showed leptospiral antigens in the brain from 12-24 hrs p.i.

Icterohaemorrhagiae and pomona were the two most frequently isolated serovars from canine leptospirosis in Southwestern Nigeria. Structural localisation of the organism within the kidney may be an indication of the stage of infection. Renal lesions of leptospirosis were present in asymptomatic reservoir hosts. In addition to the well documented renal, hepatic, and pulmonary pathology, lesions of leptospirosis were also found in several other organs.

Keywords: Leptospiral serovars, Renal pathology, Reservoir hosts

Word count = 471

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CHAPTER ONE

1.0 GENERAL INTRODUCTION

Leptospirosis is a zoonotic bacterial disease caused by pathogenic members of the genus leptospira and it is considered the most widespread zoonotic disease in the world (WHO, 1999). Majority of leptospiral infections are observed in the tropical and subtropical regions and are caused by contact with leptospire-contaminated environments during agricultural or recreational practices or when waste disposal systems are ineffective (Sperber and Schleupner, 1989). The increase in mortality rate in the canine populations all over the world has been attributed to the re-emergence of leptospirosis (Bolin, 1996). Leptospirosis is a frequent cause of death in dogs and man in the tropical zones (Ratman, 1984), but has rarely been described in dogs in Nigeria. Historically, *Leptospira interrogans* serovars *canicola* and *icterohaemorrhagiae* are known to be pathogenic in dogs. The use of vaccines containing these serovars has markedly reduced the incidence and occurrence of the disease in the dog population (Prescott, 2002). However, in recent times, various strains of pathogenic leptospira organisms have been isolated from dogs such as *L. pomona*, *L. grippotyphosa* and *L. bratislava* in different parts of the world (McDonough, 2001). In Nigeria, knowledge of the prevalent serovars in dogs, their maintenance hosts, clinical presentation, pathogenesis and pathology is lacking.

Agunloye (2002) isolated *L. pomona* in sheep and goat and Ezeh *et al.* (1990) isolated a new serovar in cattle (*L. nigeria*). However, the prevalent strains in the dog population are unknown. Okewole and Ayoola (2009) serologically affirmed the presence of serovars other than *L. canicola* and *L. icterohaemorrhagiae* in the dog population in southwestern Nigeria. In their study, new non-vaccinal serovars of grippotyphosa, pomona and bratislava were serologically identified and these leptospiral organisms showed higher prevalence than the old vaccinal serovars of canicola and icterohaemorrhagiae. They identified various factors

such as heavy coastal rains that promote spirochetes survival in dirty flood waters, contaminating vaccination-induced "carrier" state that exposes dogs to new and more pathogenic serovars, use of abattoir offal as dog food and the fast urbanization in cities that promote more dog-wildlife contacts, which also promotes contact with more pathogenic wild serovars, as being responsible for the change in epidemiology.

In Nigeria, there is a dearth of information on human leptospirosis in the literature. Leptospirosis in humans presents with symptoms that are similar to those of other better known parasitic, viral and bacterial infections such as malaria, typhoid fever and brucellosis. Hence, it is frequently misdiagnosed and its impact on African communities is largely undocumented. In humans, the overt signs of the disease are fever, headache, myalgia, jaundice, subconjunctival suffusion, oliguria, lymph node enlargement, abdominal pain, vomiting, diarrhea, altered sensorium, neck stiffness, haematuria, breathlessness, epistaxis and petechial haemorrhages.

The initial clinical signs of leptospirosis are usually nonspecific in dogs and may include fever, depression, anorexia, stiffness, myalgia, shivering and weakness. The mucous membranes are often injected. These symptoms may be followed by signs of kidney dysfunction including anuria, haematuria or polyuria, vomiting, dehydration and oral ulceration. Abortions, diarrhea, gray stools, coughing, dyspnea, conjunctivitis, weight loss and jaundice may also be seen (Miller *et al.*, 2007). Haemorrhagic syndromes occur in some dogs in which the mucous membranes may have widespread petechial and ecchymotic haemorrhages and, in later stages of the disease, there may be haemorrhagic gastroenteritis and epistaxis. However, some dogs die peracutely without clinical signs (McDonough, 2004).

In Nigeria, leptospirosis is a grossly under-reported disease, probably due to the lack of reliable diagnostic facilities or to the non-specific clinical features of the disease, lack of awareness among physicians, difficulties in isolating the organism and serologic testing, and

underestimating the prevalence of leptospirosis. In Nigeria, although social, environmental and occupational factors offer ideal conditions for successful transmission of leptospirosis, few studies had been done on both human and canine leptospirosis. In humans, leptospirosis is often not recognized or is erroneously mistaken for other diseases with similar symptoms such as malaria and typhoid fever (Faine, 1988).

In different parts of the world, it is thought that commensal rodents are the most important source of transmission to humans and dogs (Dalu and Feresu, 1997; Machangu *et al*., 1997; WHO Leptospirosis, 2003). A recent molecular epidemiological study from Italy showed that stray dogs in kennels in western Sicily were the source of infection in humans (Vitale *et al*., 2007). Important leptospirosis reservoir hosts for dog are rats and mice living near human habitats, domestic animals such as cattle and swine, companion animals, especially dogs and cats, and wild animals especially rodents (Faine *et al*., 1999).

Despite the yearly vaccination programme against canine leptospirosis, reports from several veterinary clinics and high number of postmortem and histopathological diagnoses in dog have indicated an increase in the incidence of suspected cases of leptospirosis in dogs appropriately vaccinated against canine leptospirosis. Okewole and Ayoola (2009) also recently reported a total of 32, 45 and 54 valuable adult dogs (including referrals) that died of a severe clinical syndrome pathologically attributable to acute renal failure associated with leptospirosis in 2001, 2002 and 2003 respectively at the Small Animal Clinic of the Veterinary Teaching Hospital of University of Ibadan. These observations had led to the speculation that either the two serogroups (canicola and icterohaemorrhagiae) contained in all the vaccines used commonly in Nigeria are not responsible for most of the cases of canine leptospirosis in Nigeria or that there were vaccine failures due to improper storage temperature. But little or no empirical proofs are available to support these assertions. This background has prompted the investigation of this disease in dogs and possible reservoir

hosts such as cattle, and wild rodents, in order to improve our understanding of the epidemiology, clinical presentation, pathogenesis, pathology and diagnosis of the disease in Nigeria.

1.1 JUSTIFICATION FOR THE PRESENT STUDY

Until now, canine leptospirosis has not been given due consideration as a possible cause of mortalities in the dog population in Nigeria. In Nigeria, leptospirosis is a grossly under-reported disease, probably due to the lack of diagnostic facilities, non-specific clinical features of the disease, lack of awareness among physicians, difficulties in isolating the organism and serologic testing, and underestimation of the disease incidence. Although, prevailing social, environmental and occupational factors offer ideal conditions for successful transmission of leptospirosis, but little investigation has been done in Nigeria on both human and canine leptospirosis. Knowledge of the prevailing serovars, clinical signs, clinico-pathological profiles, pathogenesis and pathology that result in mortalities in dog population is required in order to make a correct diagnosis and evaluate the extent of damage caused by the organism in different organs.

1.2 STUDY OBJECTIVES

1.2.1 General objectives

Despite the plethora of information in the literature on canine leptospirosis, few studies have dwelt on cultural isolation and characterization of leptospiral organism with the associated pathology using different diagnostic methods such as EMJH medium, monoclonal antibodies, Warthin Starry silver stain and immunohistochemistry. Most of the available studies were based on the sero-epidemiological survey in canine populations and the asymptomatic carriers such as wild rat, and cattle (Gautam *et al.,* 2010). Therefore the aim and objectives of

this study were to 1) investigate the prevalence of leptospirosis as the cause of death in the dog population in southwest Nigeria, 2) determine the prevalence and pathology of leptospirosis in asymptomatic carriers such as cattle and wild rats which might serve as source infection to dogs, 3) study the pathogenesis and pathology of leptospirosis in a guinea pig model.

1.2.2 Specific objectives

1.2.2.1 In dogs

Most of the studies on canine leptospirosis in Nigeria have been based on serological evidence (Okewole and Ayoola, 2009). There is a dearth of information on isolation and characterization of leptospira serovars, with the associated pathology in dogs in Nigeria. Therefore, the objectives of this study are to 1) determine the prevalence of canine leptospirosis between 2003 to 2010 in the Southwest zone of Nigeria, 2) determine the effects of risk factors such as age, breed and sex on the occurrence of canine leptospirosis, 3) characterize the prevailing leptosira serovars in dogs, 4) determine the clinico-haematological and biochemical changes in naturally infected dogs, 5) determine the associated pathology of canine leptospirosis in naturally infected dogs using modern techniuques.

1.2.2.2 In Cattle

According to Barr, (2000) and Okewole and Ayoola (2009), cattle might serve as source of infection to both humans and domestic animals especially dogs when fed with abattior offals from animals in the leptospiraemic phase of the disease and most of the home-made dog foods in Nigeria are made from abattoir offals. Therefore the aim and objectives of this study are to; 1) determine the prevalence of leptospiral infection in cattle, 2) characterize the various serovars in cattle and correlate them with the prevailing serovars in dogs, 3) determine a possible underlying renal pathology associated with leptospiral infection in cattle.

1.2.2.3 In wild rats

Rodents, especially wild rats have been incriminated as the source of transmission of the disease to both humans and dogs. Therefore the aims and objectives of this study are to:

1) determine the prevalence of leptospiral infection in the wild rats, 2) characterize the various leptospira serovars in wild rats and correlate them with the prevailing serovars in dogs, 3) determine a possible underlying renal pathology associated with leptospiral infection in wild rats.

1.2.2.4. In a guinea pig model

The aims and objectives of experimental leptospiral infection in a guinea pig model were to

1) Determine the pathogenesis of leptospiral infection in tissues and different organs of infected guinea pigs, and to elucidate the reason why the leptospira organism are not detected in some organs of dogs examined post-mortem, except liver and kidney. 2) Determine the gross and histopathological changes in different organs of infected guinea pigs. 3) Compare the pathology of infected guinea pigs with those observed in dogs

CHAPTER TWO

2.0: LITERATURE REVIEW

2.1: HISTORICAL ASPECTS

Leptospirosis is a zoonosis of ubiquitous distribution, caused by infection with pathogenic *Leptospira* species. The spectrum of human and animal diseases caused by leptospires is extremely wide, ranging from subclinical infection to a severe syndrome of multiorgan damage with high mortality. This syndrome, icteric leptospirosis with renal failure, was first reported over 100 years ago by Adolf Weil in Heidelberg (Weil, 1886). However, an apparently identical syndrome occurring in sewer workers was described several years earlier (Landouzy, 1883a). Earlier descriptions of diseases that were probably leptospirosis were reviewed recently (Faine, 1994; Everard, 1996).

Leptospirosis was recognized as an occupational hazard of rice harvesting in ancient China (Faine, 1994), and the Japanese name Akiyami, or autumn fever, persists in modern medicine. With hindsight, clear descriptions of leptospiral jaundice can be recognized as having appeared earlier in the 19th century, some years before the description by Weil (Faine, 1994). It has been suggested that *Leptospira interrogans* serovar icterohaemorrhagiae was introduced to Western Europe in the 18th century by westward extension of the range of household rats (*Rattus norvegicus)* from Eurasia (Alston and Broom, 1958).

The aetiology of leptospirosis was demonstrated independently in 1915 in Japan and Germany (Everard, 1996). In Japan, Inada and Ido*et al* (1917) detected both spirochetes and specific antibodies in the blood of Japanese miners with infectious jaundice, and two groups of German physicians studied German soldiers afflicted by “French disease” in the trenches of northeast France. Uhlenhuth and Fromme, (1915) and Hubener and Reiter, (1915) detected spirochetes in the blood of guinea pigs inoculated with the blood of infected soldiers.

Unfortunately, these two groups became so embroiled in arguments over priority that they overlooked the first publications in English (Inada *et al.*, 1916) and German of papers by Inada's group, whose initial publications predated their own by 8 months. Confirmation of the occurrence of leptospirosis on both sides of the Western Front was obtained rapidly after the publication in Europe of Inada's work (Costa and Troisier, 1916; Dawson and Hume, 1916 and Stokes *et al.*, 1917).

Given the initial controversy over nomenclature, it is ironic that the organism had first been described almost 10 years before (Stimson, 1907). Stimson demonstrated by silver staining the presence of clumps of spirochetes in the kidney tubules of a patient who reportedly died of yellow fever. The spirochetes had hooked ends, and Sti``mson named them *Spirochaeta interrogans* because of their resemblance to a question mark. Unfortunately, this sentinel observation was overlooked for many years (Faine, 1994).

The importance of occupation as a risk factor was recognized early. The role of the rat as a source of human infection was discovered in 1917 (Ido *et al.*, 1917), while the potential for leptospiral disease in dogs was recognized, but clear distinction between canine infection with *L. interrogans* serovars icterohaemorrhagiae and canicola took several years (Klarenbeek and Schuffner,1933). Leptospirosis in livestock was recognized some years later (Alston and Broom, 1958). Several monographs provide extensive information on the early development of knowledge on leptospirosis (Wolff 1954; Alston and Broom, 1958; Faine, 1994; Faine *et al.*, 1999).

2.2. BACTERIOLOGY

2.2.1. Taxonomy and Classification

2.2.1.1. Serological classification

Prior to 1989, the genus *Leptospira* was divided into two species, *L. interrogan*s, comprising all pathogenic strains and *L. biflex*a, containing the saprophytic strains isolated from the environment (Faine and Stallman, 1982). *L. biflexa* was differentiated from *L. interrogans* by the growth of the former at 13°C and growth in the presence of 8-azaguanine (225 mg/ml) and by the failure of *L. biflexa* to form spherical cells in 1M NaCl.

Both *L. interrogans* and *L. biflexa* are divided into numerous serovars defined by agglutination after cross-absorption with homologous antigen (Dikken and Kmety, 1978; Johnson and Faine, 1984; Kmety and dikken, 1993). If more than 10% of the homologous titer remains in at least one of the two antisera on repeated testing, two strains are said to belong to different serovars (International Committee on Systematic Bacteriology Subcommittee on Taxonomy of *leptospira*, 1987). Over 60 serovars of *L. biflexa* have been recorded (Johnson and Faine, 1984). Within the species *L. interrogans* over 200 serovars are recognized; additional serovars have been isolated but have yet to be validly published. Serovars that are antigenically related have traditionally been grouped into serogroups (Dikken and Kmety, 1993). While serogroups have no taxonomic standing, they have proved useful for epidemiological understanding.

2.2.1.2. Genotypic classification

The phenotypic classification of leptospires has been replaced by a genotypic one, in which a number of genomospecies include all serovars of both *L. interrogans* and *L. biflex*a. Genetic heterogeneity was demonstrated some time ago (Haapala *et al.*, 1969; Brendle *et al.*, 1974), and DNA hybridization studies led to the definition of 10 genomospecies of *Leptospira*

(Yasuda *et al.*, 1987). An additional genomospecies, *L. kirschneri*, was added later (Ramadass *et al.*, 1992).

After an extensive study of several hundred strains, workers at the Centers for Disease Control (CDC) more recently defined 16 genomospecies of *Leptospira* that included those described previously (Ramadass *et al.*, 1992) and adding five new genomospecies (Brenner *et al.*, 1991), one of which was named *L. alexanderi*. An additional species, *L. fainei*, has since been described, which contains a new serovar, hurstbridge (Perolat *et al.*, 1990). DNA hybridization studies have also confirmed the taxonomic status of the mono-specific genus *Leptonema* (Yasuda *et al.*, 1987; Brenner *et al.*, 1991). The genotypic classification of leptospires is supported by multilocus enzyme electrophoresis data (Letocart *et al.*, 1997), but recent studies suggest that further taxonomic revisions are likely (Letocart *et al.*, 1999; Postic *et al.*, 2000).

The genomospecies of *Leptospira* do not correspond to the previous two species *(L. interrogans* and *L. biflexa*), and indeed, pathogenic and nonpathogenic serovars occur within the same species. Thus, neither serogroup nor serovar reliably predicts the species of *Leptospira*. Moreover, recent studies (Brenner *et al.*, 1991; Feresu *et al.*, 1999) have included multiple strains of some serovars and demonstrated genetic heterogeneity within serovars. In addition, the phenotypic characteristics formerly used to differentiate *L. interrogans* sensu lato from *L. biflexa* sensu lato do not differentiate the genomospecies (Ramadass *et al.*, 1992).

The reclassification of leptospires on genotypic grounds is taxonomically correct and provides a strong foundation for future classifications. However, the molecular classification is problematic for the clinical microbiologist, because it is clearly incompatible with the system of serogroups which has served clinicians and epidemiologists well for many years. Until simpler DNA-based identification methods are developed and validated, it will be

necessary for clinical laboratories to retain the serological classification of pathogenic leptospires for the foreseeable future. In addition, the retention of *L. interrogans* and *L. biflexa* as specific names in the genomic classification also allows nomenclatural confusion. In the following pages, specific names refer to the genomospecies, including *L. inter-rogans* sensu stricto and *L. biflexa* sensu stricto.

2.2.2. Biology of Leptospires

Leptospires are tightly coiled spirochetes, usually 0.1 mm by 6 to 0.1 by 20 mm, but occasional cultures may contain much longer cells. The helical amplitude is approximately 0.1 to 0.15mm, and the wavelength is approximately 0.5 mm (Faine *et al.*, 1999). The cells have pointed ends, either or both of which are usually bent into a distinctive hook. Two axial filaments (periplasmic flagella) with polar insertions are located in the periplasmic space (Swain, 1957). The structure of the flagellar proteins is complex (Trueba *et al.*, 1995). Leptospires exhibit two distinct forms of movement, translational and nontranslational (Berg *et al.*, 1978). Morphologically all leptospires are indistinguishable, but the morphology of individual isolates varies with subculture in vitro and can be restored by passage in hamsters (Ellis *et al.*, 1986).

Leptospires have a typical double membrane structure in common with other spi-rochetes, in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and are overlain by an outer membrane (Haake *et al.*, 1993). Leptospiral lipopolysaccharide has a composition similar to that of other gram-negative bacteria (Vinh *et al.*, 1986), but has lower endotoxic activity (Shimizu *et al*, 1987). Leptospires may be stained using carbol fuchsin counterstain (Faine, 1994).

Leptospires are obligate aerobes with an optimum growth temperature of 28 to 30°C. They produce both catalase and oxidase (Simbert, 1977). They grow in simple media

enriched with vitamins (vitamins B2 and B12 are growth factors), long-chain fatty acids, and ammonium salts (Johnson and Faine, 1984). Long-chain fatty acids are utilized as the sole carbon source and are metabolized by b-oxidation (Smibert, 1977).

2.2.3. Culture Methods

Growth of leptospires in media containing serum or albumin plus polysorbate and in protein-free synthetic media has been described (Turner, 1970). Several liquid media containing rabbit serum were described by Fletcher, Korthoff, Noguchi, and Stuart (Turner, 1970); recipes for these earlier media are found in several monographs (Faine *et al.*, 1999). The most widely used medium in current practice is based on the oleic acid-albumin medium EMJH (Ellinghausen and McCullough, 1965; Johnson and Harris, 1967). This medium is available commercially from several manufacturers and contains Tween 80 and bovine serum albumin. Some strains are more fastidious and require the addition of either pyruvate (Johnson *et al.*, 1973) or rabbit serum (Ellis *et al.*, 1986) for initial isolation. Growth of contaminants from clinical specimens can be inhibited by the addition of 5-fluorouracil (Knight *et al.*, 1973). Other antibiotics have been added to media for culture of veterinary specimens, in which contamination is more likely to occur (Adler *et al.*, 1986; Myer and Varela-Diaz, 1973). Protein-free media have been developed for use in vaccine production (Schuffner, 1983).

Growth of leptospires is often slow on primary isolation, and cultures are retained for up to 13 weeks before being discarded, but pure subcultures in liquid media usually grow within 10 to 14 days. Agar may be added at low concentrations (0.1 to 0.2%). In semisolid media, growth reaches a maximum density in a discrete zone beneath the surface of the medium, which becomes increasingly turbid as incubation proceeds. This growth is related to the optimum oxygen tension (Faine *et al.*, 1999) and is known as a Dinger's ring or disk

(Dinger, 1932). Leptospiral cultures may be maintained by repeated subculture (Vinh *et al.*, 1986) or preferably by storage in semisolid agar containing hemoglobin (Faine *et al.*, 1999). Long-term storage by lyophilization (Annear, 1974) or at 270°C (Palit *et al.*, 1986) is also used.

Growth on media solidified with agar has been reported (Turner, 1970). Colonial morphology is dependent on agar concentration and serovar (Tripathy *et al.*, 1980). Media can also be solidified using gellan gum (Rule and Alexander, 1986). Solid media have been used for isolation of leptospires (Thiermann, 1981), to separate mixed cultures of leptospires, and for detection of hemolysin production (Stamm and Charon, 1979).

2.2.4. Molecular Biology

Leptospires are phylogenetically related to other spirochetes (Paster *et al.*, 1991). The leptospiral genome is approximately 5,000 kb in size (Baril and Saint, 1990; Zuerner, 1991), although smaller estimates have been reported (Xiao *et al.*, 1990; Taylor *et al.*, 1991). The genome is comprised of two sections, a 4,400-kb chromosome and a smaller 350-kb chromosome (Zuerner, 1991). Physical maps have been constructed from serovars pomona subtype kennewicki (Zuerner, 1991) and icterohaemorrhagiae (Boursaux-Eude *et al.*, 1998; Takahashi *et al.*, 1998). Leptospires contain two sets of 16S and 23S rRNA genes but only one 5S rRNA gene (Fukunaga and Mifuchi, 1989a), and the rRNA genes are widely spaced (Fukunaga and Mifuchi, 1989b; Baril *et al.*, 1990).

The study of leptospiral genetics has been slowed by the lack of a transformation system (Kalambahete *et al.*, 1999). Recently, a shuttle vector was developed using the temperate bacteriophage LE1 from *L. biflexa* (Saint Girons *et al.*, 2000). This advance offers the prospect of more rapid progress in the understanding of *Leptospira* at the molecular level.

Several repetitive elements have been identified (Boursaux-Eude *et al.*, 1995; Kalambahete *et al.*, 1999), of which several are insertion sequences (IS) coding for transposases. I*S1533* has a single open reading frame (Zuerner, 1994), while I*S1500* has four. Both I*S1500* and I*S1533* are found in many serovars (Zuerner and Bolin, 1990; Kalambahete *et al.*, 1999), but the copy number varies widely between different serovars and among isolates of the same serovar (Boursaux-Eude *et al.*, 1998). A role for these insertion sequences in transposition and genomic rearrangements has been identified (Zuerner, 1994, Boursaux-Eude *et al.*, 1998). Other evidence for horizontal transfer within the genus *Leptospira* has been reported (Ralph and McClelland, 1994).

A number of leptospiral genes have been cloned and analyzed, including several for amino acid synthesis (Zuerner and Charon, 1988; Richard *et al.*, 1990; Ding and Yelton, 1993), rRNA (Fukunaga *et al.*, 1990; Fukunaga *et al.*, 1991), ribosomal proteins (Zuerner *et al.*, 2000), RNA polymerase (Renesto *et al.*, 2000), DNA repair (Stamm *et al.*, 1991), heat shock proteins (Ballard *et al.*, 1993; Park *et al.*, 1999), sphingomyelinase (Segers *et al.*, 1990; Segers *et al.*, 1992), hemolysins (Lee *et al.*, 2000), outer membrane proteins (Haake *et al.*, 2000), flagellar proteins (Trueba *et al.*, 1995; Lin *et al.*, 1999), and lipopolysaccharide (LPS) synthesis (kalambaheti *et al.*, 1999; Bulach *et al.*, 2000).

Within serovar icterohaemorrhagiae, the genome appears to be conserved (Takahashi *et al.*, 1998). This conservation allowed the identification of at least one new serovar by recognition of distinct pulsed-field gel electrophoresis (PFGE) profiles (Herrmann *et al.*, 1994). However, the recent demonstration of heterogeneity within serovars (Feresu *et al.*, 1999) indicates the need for further study of multiple isolates of individual serovars.

2.3. EPIDEMIOLOGY

Leptospirosis is presumed to be the most widespread zoonosis in the world (WHO, 1999). The source of infection in humans is usually either direct or indirect contact with the urine of an infected animal. The incidence is significantly higher in warm-climate countries than in temperate regions (Everard and Everard, 1993); this is due mainly to longer survival of leptospires in warm, humid conditions. However, most tropical countries are also developing countries, and there are greater opportunities for exposure of the human population to infected animals, whether livestock, domestic pets, or wild or feral animals. The disease is seasonal, with peak incidence occurring in summer or fall in temperate regions, where temperature is the limiting factor to survival of leptospires, and during rainy seasons in warm-climate regions, where rapid desiccation would otherwise prevent survival.

The reported incidence of leptospirosis reflects the availability of laboratory diagnosis and the clinical index of suspicion as much as the incidence of the disease. Within the United States, the highest incidence is found in Hawaii (Centers for Disease Control and prevention, 1994). Leptospirosis ceased to be a notifiable infection within the United States after December 1994 (Centers for Disease Control and Prevention, 1998).

The usual portal of entry is through abrasions or cuts in the skin or via the conjunctiva; infection may take place via intact skin after prolonged immersion in water, but this usually occurs when abrasions are likely to occur and is thus difficult to substantiate. Water-borne transmission has been documented; point contamination of water supplies has resulted in several outbreaks of leptospirosis. Inhalation of water or aerosols may also result in infection via the mucous membranes of the respiratory tract. Rarely, infection may follow animal bites (Gollop *et al.*, 1993). Direct transmission between humans has been demonstrated rarely. However, excretion of leptospires in human urine months after recovery has been recorded (Bal *et al.*, 1994). It is thought that the low pH of human urine limits

survival of leptospires after excretion. Transmission by sexual intercourse during convalescence has been reported (Harrison and Fitzgerald, 1988).

Animals, including humans, can be divided into maintenance hosts and accidental (incidental) hosts. The disease is maintained in nature by chronic infection of the renal tubules of maintenance hosts (Babudieri, 1958). A maintenance host is defined as a species in which infection is endemic and is usually transferred from animal to animal by direct contact. Infection is usually acquired at an early age, and the prevalence of chronic excretion in the urine increases with the age of the animal. Other animals (such as humans) may become infected by indirect contact with the maintenance host. Animals may be maintenance hosts of some serovars but incidental hosts of others, infection with which may cause severe or fatal disease. The most important maintenance hosts are small mammals, which may transfer infection to domestic farm animals, dogs, and humans.

The extent to which infection is transmitted depends on many factors, including climate, population density, and the degree of contact between maintenance and accidental hosts. Different rodent species may be reservoirs of distinct serovars, but rats are generally maintenance hosts for serovars of the serogroups lcterohaemorrhagiae and Ballum, and mice are the maintenance hosts for serogroup Ballum. Domestic animals are also maintenance hosts; dairy cattle may harbor serovars Hardjo, Pomona, and Grippotyphosa; pigs may harbor pomona, tarassovi, or bratislava; sheep may harbor hardjo and pomona; and dogs may harbor canicola (Bolin, 2000). Distinct variations in maintenance hosts and the serovars they carry occur throughout the world (Hartskeerl and Terpstra, 1996). Knowledge of the prevalent serovars and their maintenance hosts is essential to understanding the epidemiology of the disease in any region.

Human infections may be acquired through occupational, recreational, or vocational exposures. Occupation is a significant risk factor for humans (Waitkins, 1984). Direct contact

with infected animals accounts for most infections in farmers, veterinarians, abattoir workers (Campagnolo *et al.*, 2000), meat inspectors (Blackmore, et al., 1979), rodent control workers (Demers *et al.*, 1985), and other occupations which require contact with animals (Looke, 1986). Indirect contact is important for sewer workers, miners, soldiers (Johnson *et al.*, 1983), septic tank cleaners, fish farmers (Robertson *et al.*, 1981), gamekeepers, canal workers (Andre-Fontaine,*et al.*, 1992), rice field workers (Padre *et al.*, 1988), taro farmers (Anderson and Minette, 1986), banana farmers (Smythe *et al.*, 2000), and sugar cane cutters (Cotter, 1936).

The occurrence of Weil's disease in sewer workers was first reported in the 1930s (Stuart, 1939). Serovar icterohaemorrhagiae was isolated by guinea pig inoculation from patients, from rats trapped in sewers (Johnson, 1937). In Glasgow, Scotland, a seroprevalence among sewer workers of 17% was reported (Stuart, 1939). The recognition of this important risk activity led to the adoption of rodent control programs and the use of protective clothing, resulting in a significant reduction in cases associated with this occupation. The presence in wastewater of detergents is also thought to have reduced the survival of leptospires in sewers (Waitkins, 1986), since leptospires are inhibited at low detergent concentrations.

Fish workers were another occupational group whose risk of contracting leptospirosis was recognized early. Between 1934 and 1948, 86% of all cases in the northeast of Scotland oc-curred in fish workers in Aberdeen (Smith, 1949). Recognition of risk factors and adoption of both preventive measures and rodent control have reduced the incidence of these occupational infections greatly. From 1933 to 1948 in the British Isles, there were 139 cases in coal miners, 79 in sewer workers, and 216 in fish workers.

However, in the period from 1978 to 1983, there were nine cases in these three occupations combined (Waitkins, 1984). More recently, fish farmers have been shown to be at risk (Robertson *et al.*, 1981), particularly for infection with serovars of serogroup

Icterohaemorrhagiae (Gill, *et al.*, 1985a), presumed to be derived from rat infestation of premises. Because of the high mortality rate associated with Icterohaemorrhagiae infections, this was considered an important occupational risk group despite the very small absolute number of workers affected (Gill, *et al.*, 1985b).

Livestock farming is a major occupational risk factor throughout the world. The highest risk is associated with dairy farming and is associated with serovar hardjo (Waitins, 1986), in particular with milking of dairy cattle. Human cases can be associated with clinical disease in cattle, but are not invariably so. Cattle are maintenance hosts of serovar hardjo (Ellis, *et al.*, 2000), and infection with this serovar occurs throughout the world. Many animals are seronegative carriers (Thiermann, 1983).

After infection, leptospires localize in the kidneys (Postic *et al.*, 2000) and are excreted intermittently in the urine (Ellis and Michna, 1977). Serovar hardjo causes outbreaks of mastitis and abortion. Serovar hardjo is found in aborted fetuses and in premature calves (Ellis *et al.*, 2000). In addition, hardjo has been isolated from normal fetuses (Ellis *et al.*, 1982), the genital tracts of pregnant cattle (Ellis *et al.*, 1982), vaginal discharge after calving, and the genital tract and urinary tract of .50% of cows and bulls (Ellis *et al.*, 1985). In Australia, both serovars hardjo and Pomona were demonstrated in bovine abortions, but serological evidence suggested that the incidence of hardjo infection was much higher (Elder *et al.*, 1985). In Scotland, 42% of cattle were seropositive for hardjo, representing 85% of all seropositive animals (Ellis and Michna, 1976a). Ellis and Thiermann, (1986), reported serovar hardjo has been the most commonly isolated serovar in cattle in the United States, but pomona also occurs.

Several outbreaks of leptospirosis associated with water have been reported. Many of these outbreaks have followed extended periods of hot, dry weather, when pathogenic

leptospires presumably have multiplied in freshwater ponds or rivers. Cases of leptospirosis also follow extensive flooding (Fuortes and Nettleman, 1994).

Pathogenic serovars have been isolated from water in tropical regions (Alexander, *et al.*, 1975). Survival of pathogenic leptospires in the environment is dependent on several factors, including pH, temperature, and the presence of inhibitory compounds. Most studies have used single serovars and quite different methodologies, but some broad conclusions may be drawn. Under laboratory conditions, leptospires in water at room temperature remain viable for several months at pH 7.2 to 8.0 (Gordon-Smith and Turner, 1961), but in river water survival is shorter and is prolonged at lower temperatures (Crawford *et al.*, 1971).

The presence of domestic sewage decreases the survival time to a matter of hours (Chang, *et al.*, 1958), but in an oxidation ditch filled with cattle slurry, viable leptospires were detected for several weeks (Diesch, 1971). In acidic soil (pH 6.2) taken from canefields in Australia, serovar australis survived for up to 7 weeks, and in rainwater-flooded soil it survived for at least 3 weeks. When soil was contaminated with urine from infected rats or voles, leptospires survived for approximately 2 weeks (Karazeva, 1973). In slightly different soil, serovar pomona survived for up to 7 weeks under conditions approximating the New Zealand winter (Hellstrom and Marshall, 1978).

Many sporadic cases of leptospirosis in tropical regions are acquired following vocational exposures that occur during the activities of daily life (Everard *et al.*, 1992). Dogs are a significant reservoir for human infection in many tropical countries (Weekes, *et al.*, 1997) and may be an important source of outbreaks. A number of outbreaks of leptospirosis have resulted from contamination of drinking water and from handling rodents (Ellis *et al.*, 2000).

Faine, 1994, defined three epidemiological patterns of leptospirosis. The first occurs in temperate climates where few serovars are involved and human infection almost invariably

occurs by direct contact with infected animals through farming of cattle and pigs. Control by immunization of animals and/or humans are potentially possible. The second occurs in tropical wet areas, within which there are many, more serovars infecting humans and animals and larger numbers of reservoir species, including rodents, farm animals, and dogs. Human exposure is not limited by occupation but results more often from the widespread environmental contamination, particularly during the rainy season. Control of rodent populations, drainage of wet areas, and occupational hygiene are all necessary for prevention of human leptospirosis. The third pattern comprises rodent-borne infection in the urban environment. While this is of lesser significance throughout most of the world, it is potentially more important when the urban infrastructure is disrupted by war or by natural disasters.

2.4. CLINICAL FEATURES OF LEPTOSPIROSIS

Leptospirosis has been described as a zoonosis of protean manifestations (Peter, 1982). Indeed, this description has been so overused as to have become a cliche ´. The spectrum of symptoms is extremely broad; the classical syndrome of Weil's disease represents only the most severe presentation. Formerly it was considered that distinct clinical syndromes were associated with specific serogroups (Van Thiel, 1948). However, this view was questioned by some authorities (Alexander *et al.*, 1963; Feigin and Anderson, 1975), and more intense study over the past 30 years has refuted this hypothesis. An explanation for many of the observed associations may be found in the ecology of the maintenance animal hosts in a geographic region.

The clinical presentation of leptospirosis is biphasic, with the acute or septicemic phase lasting about a week, followed by the immune phase, characterized by antibody production and excretion of leptospires in the urine (Turner, 1967; Kelley, 1998). Most of the

complications of leptospirosis are associated with localization of leptospires within the tissues during the immune phase and thus occur during the second week of the illness.

2.4.1. Anicteric Leptospirosis

The great majority of infections caused by leptospires are either subclinical or of very mild severity, and patients will probably not seek medical attention. A smaller proportion of infections, but the overwhelming majority of the recognized cases, present with a febrile illness of sudden onset. Other symptoms include chills, headache, myalgia, abdominal pain, conjunctival suffusion, and less often a skin rash. If present, the rash is often transient, lasting less than 24 hours.

This anicteric syndrome usually lasts for about a week, and its resolution coincides with the appearance of antibodies. The fever may be biphasic and may recur after a remission of 3 to 4 days. The headache is often severe, resembling that occurring in dengue, with retro-orbital pain and photophobia. Myalgia affecting the lower back, thighs, and calves is often intense (Alexander *et al.*, 1963; Kelley, 1998).

Aseptic meningitis may be found in ≤ 25% of all leptospirosis cases and may account for a significant minority of all causes of aseptic meningitis (Schaeffer, 1951; Beeson and Hankey, 1952; King and Urguhart, 1975). Patients with aseptic meningitis have tended to be younger than those with icteric leptospirosis (Schaeffer, 1951; Beeson and Hankey, 1952; King and Urguhart, 1975). In the series of 616 cases, Alston and Broom (1958) noted that 62% of children ≤ 14 years old presented with aseptic meningitis, whereas only 31% of patients aged 15 to 29 years did so and only 10% of those over 30 years of age. Mortality is almost nil in anicteric leptospirosis, but death resulting from massive pulmonary hemorrhage occurred in 2.4% of the anicteric patients in a Chinese outbreak (Wang *et al.*, 1965).

The differential diagnosis must include common viral infections, such as influenza (Alexander *et al.,* 1963) and, in the tropics, dengue (Sanders *et al.*, 1999), in addition to the bacterial causes of fever of unknown origin, such as typhoid. Turner (1967) provided a comprehensive list of other conditions that may be mimicked by leptospirosis, including encephalitis, poliomyelitis, rickettsiosis, glandular fever (infectious mononucleosis), brucellosis, malaria, viral hepatitis, and pneumonitis. Hantavirus infections must also be considered in the differential diagnosis for patients with pulmonary involvement. Petechial or purpuric lesions may occur (Chang *et al.*, 1958; Alexander *et al.*, 1963), and recently, cases of leptospirosis resembling viral hemorrhagic fevers have been reported in travelers returning from Africa (Heron *et al.*, 1997; Monseuz, 1997).

2.4.2. Icteric Leptospirosis

Icteric leptospirosis is a much more severe disease in which the clinical course is often very rapidly progressive. The jaundice occurring in leptospirosis is not associated with hepatocellular necrosis, and liver function returns to normal after recovery (Ramos-Morale *et al.*, 1959). Serum bilirubin levels may be high, and many weeks may be required for normalization (Edwards *et al.*, 1990). There are moderate rises in transaminase levels, and minor elevation of the alkaline phosphatase level usually occurs.

The complications of severe leptospirosis emphasize the multisystemic nature of the disease. Leptospirosis is a common cause of acute renal failure (ARF), which occurs in 16 to 40% of cases (Edwards *et al.*, 1990; Abdulkader, 1997). A distinction may be made between patients with prerenal azotemia (non-ARF) and those with ARF. Patients with prerenal azotaemia may respond to rehydration, and decisions regarding dialysis can be delayed for up to 72 h (Nicholson, 1989). In patients with ARF, oliguria was a significant predictor of death (Dai *et al.,* 1994).

Serum amylase levels are often raised significantly in association with ARF (Alexander *et al.*, 1963; Edwards and Everard, 1991; O'brien *et al.*, 1998), but clinical symptoms of pancreatitis are not a common finding (Edward, 1999). Necrotizing pancreatitis has been detected at autopsy (Edwards and Everard, 1991). Thrombocytopenia (platelet count of <100 X 10^9 /liter) occurs in ≥50% of cases and is a significant predictor for the development of ARF (Edwards, *et al.*, 1982). However, thrombocytopenia in leptospirosis is transient and does not result from disseminated intravascular coagulation (Edwards *et al.*, 1986; Nicodemo *et al.*, 1997).

2.4.3. Pulmonary involvement

The occurrence of pulmonary symptoms in cases of leptospirosis was first noted by Silverstein (1953). Subsequent reports have shown that pulmonary involvement may be the major manifestation of leptospirosis in some clusters of cases (Wang, *et al.*, 1965; Sehgal, *et al.*, 1995 and Zaki *et al.*, 1996) and in some sporadic cases. The severity of respiratory disease is unrelated to the presence of jaundice (Im *et al.*, 1989; Hill and Sanders, 1997). Patients may present with a spectrum of symptoms, ranging from cough, dyspnea, and hemoptysis (which may be mild or severe) to adult respiratory distress syndrome (Alani *et al.*, 1993; Emmanouilides, *et al.*, 1994). Intra-alveolar hemorrhage was detected in the majority of patients, even in the absence of overt pulmonary symptoms (du Coudedic *et al.*, 1998). Pulmonary hemorrhage may be severe enough to cause death (Trevejo *et al.*, 1998; Yersin, 2000).

The incidence of respiratory involvement varies. In a Chinese series of anicteric cases, more than half had respiratory symptoms, while 67% had radiographic changes (Wang, *et al.*, 1965); in a similar Korean series, 67% of patients had respiratory symptoms and 64% had radiographic abnormalities (Im *et al.*, 1989), whereas in a series of jaundiced patients in Brazil, only 17% had clinical evidence of pulmonary involvement, but 33% had radiographic

abnormalities (Nery *et al.*, 1977). Rales are more common in icteric than in nonicteric leptospirosis (Alexander *et al.*, 1963). Concurrent hemoptysis and pulmonary infiltrates on chest radiographs were noted in 12% of 69 nonfatal cases in the Seychelles (Yasuda *et al.*, 1987).

Radiography generally reveals diffuse small opacities which may be widely disseminated or which may coalesce into larger areas of consolidation, with increasing severity of symptoms (Zaki *et al.*, 1996; Yersin *et al.*, 2000). Pleural effusions may occur (Lee *et al.*, 1986; Teglia *et al.*, 1995). The patchy infiltrates which are commonly seen reflect areas of intra-alveolar and interstitial hemorrhage (Zaki *et al.*, 1996; Nicodemo *et al.*, 1997). Both alveolar infiltrates and dyspnea are poor prognostic indicators in severe leptospirosis (Dupont *et al.*, 1997). Similarly, in icteric leptospirosis in Brazil, respiratory insufficiency was associated with death (Ko *et al.*, 1999).

2.4.4. Cardiac Involvement

Cardiac involvement in leptospirosis is common but may be underestimated. Fatal myocarditis was first described in 1935 by Mollaret and ferroir. Clinical evidence of myocardial involvement, including abnormal T waves, was detected in 10% of 80 severe icteric cases in Louisiana (Sodeman and Killough, 1951), while similar electrocardiographic (ECG) abnormalities were detected in over 40% of patients in China, India, Sri Lanka, and the Philippines (Watt *et al.*, 1990; Rajiv *et al.*, 1996), including both icteric and nonicteric cases.

However, in a prospective study in Malaysia, identical ECG changes were found in patients with either leptospirosis or malaria (Parson, 1965), and it was concluded that such ECG changes were nonspecific. The presence of myocarditis was strongly associated with the severity of pulmonary symptoms in anicteric Chinese patients (Lin *et al.*, 1965). A mortality

rate of 54% was reported in severe leptospirosis cases with myocarditis (Lee *et al.*, 1986). Repolarization abnormalities on ECG were considered a poor prognostic indicator in severe leptospirosis cases (Dupont *et al.*, 1997), as were arrhythmias in a Brazilian series (Ko *et al.*, 1999).

2.4.5. Ocular Involvement

Ocular manifestations of severe leptospirosis were noted in early reports (Weekers and Firket, 1916). Conjunctival suffusion in the presence of scleral icterus is said to be pathognomonic of Weil's disease (Van Thiel, 1948). Anterior uveitis, either unilateral or bilateral, occurs after recovery from the acute illness in a minority of cases (Barkay and Garzozi, 1984). Uveitis may present weeks, months, or occasionally years after the acute stage.

In most cases uveitis is presumed to be an immune phenomenon, but leptospires have been isolated from equine eyes (Faber *et al.*, 2000), and more recently, leptospiral DNA has been demonstrated in aqueous humor by PCR (Merien *et al.*, 1993; Chu *et al.*, 1998; Faber *et al.*, 2000). Late-onset uveitis may result from an autoimmune reaction to subsequent exposure (Faine, 1994).

2.4.6. Clinical signs in different species of animals

Leptospira infections may be asymptomatic, mild or severe, and acute or chronic. The clinical signs are often related to kidney disease, liver disease or reproductive dysfunction. Chronically infected animals are often asymptomatic.

2.4.6.1 Cattle

Acute leptospirosis occurs mainly in calves. The symptoms may include fever, anorexia, conjunctivitis and diarrhea. Severely affected animals may also develop jaundice, hemoglobinuria, anemia, pneumonia, or signs of meningitis such as incoordination, salivation and muscle rigidity. Some calves may die within 3 to 5 days, and the survivors can be unthrifty after recovery and may continue to shed the organism for its entire life (Colagross-Schouten *et al.*, 2002).

The clinical signs vary with the serovar: infections with serovar *hardjo*, for example, are not usually associated with hemolytic anemia. In adult cattle, the early symptoms such as fever and depression are often transient and milder, and may go unnoticed (Corney B, 2004). The most prominent signs of infection are abortions, decreased fertility or decreased milk yield. Some serovars cause late term abortions, stillbirths and increased neonatal mortality (Alonso-Andicoberry *et al.*, 2001).

The placenta is retained in up to 20% of the cows that abort, and infertility may be a sequela. Some serovars can cause sudden agalactia or decreased milk production (Alonso-Andicoberry *et al.*, 2001). The milk may be thick, yellow, and blood-tinged but there is typically little evidence of mammary inflammation (Mahmoud *et al.*, 2008). The appearance of the milk usually improves in 4 to 5 days, and milk production returns to normal after 10 to 21 days (Mahmoud *et al.*, 2008). Jaundice may be seen in severely affected animals.

2.4.6.2 Sheep and goats

Leptospirosis in sheep and goats is similar to the disease in cattle. It is characterized by fever and anorexia and, in some animals, jaundice, hemoglobinuria or anemia. Abortions, stillbirths, weak lambs or kids and infertility can also be seen, either with or without other clinical signs. Clinical disease is relatively uncommon in sheep.

2.4.6.3 Swine

In swine, clinical leptospirosis is most often characterized by reproductive signs including late term abortions, infertility, stillbirths, mummified or macerated fetuses, and increased neonatal mortality. Fever, decreased milk production and jaundice may also be seen. In some infected herds, the only sign of infection may be a transient fever. Subclinical infections are common. In piglets, there may be fever, anorexia, depression, diarrhea, jaundice, hemoglobinuria and gastrointestinal disorders, as well as signs of meningitis. Affected piglets may grow more slowly than normal and high mortality rates can be seen in young or weak piglets.

2.4.6.4 Horses

Many infections in horses are subclinical. Ocular disease is the most common syndrome. During the acute phase, ocular signs may include fever, photophobia, conjunctivitis, miosis and iritis. Corneal opacity and periodic ophthalmia may be sequelae of acute infections. In the chronic phase, there may be anterior and posterior adhesions of the eye, a turbid vitreous body, cataracts, uveitis and other ocular abnormalities. Although systemic disease is uncommon, severe cases of leptospirosis accompanied by liver, kidney or cardiovascular disease have been described. Recently, leptospirosis has also been associated with a number of abortions.

2.4.6.5 Dogs

The clinical signs and severity of disease are highly variable in dogs. Some infections are symptomatic or mild, while others are severe or fatal. The initial signs are usually nonspecific and may include fever, depression, anorexia, stiffness, myalgia, shivering and weakness (McDonough, 2004). The mucus membranes are often injected. These symptoms may be followed by signs of kidney disease including anuria, hematuria or polyuria, vomiting, dehydration and oral ulceration. Abortions, diarrhea, gray stools, coughing,

dyspnea, conjunctivitis, weight loss and jaundice may also be seen. Hemorrhagic syndromes occur in some dogs: the mucus membranes may have widespread petechial and ecchymotic hemorrhages and, in later stages of the disease, there may be hemorrhagic gastroeneteritis and epistaxis. Some dogs die peracutely without clinical signs.

Chronic kidney disease can be a sequela. Chronic infections may be asymptomatic, or associated with fever of unknown origin and conjunctivitis. Some serovars are more likely to cause certain syndromes. Fever, hemorrhage, anemia and jaundice are typically associated with the serovar *icterohaemorrhagiae*. Serovar *grippotyphosa* tends to cause severe acute kidney failure and/or chronic active hepatitis Dogs infected with serovar *pomona* are often asymptomatic and chronic carriers Serovar *canicola* often causes chronic interstitial nephritis.

2.4.6.6 Wild animals

Infections are often asymptomatic in wild animals, including rodents.

2.4.7. Chronic or Latent Infection

Anecdotal reports suggest that leptospirosis may induce chronic symptoms analogous to those produced by other spirochetal infections, such as Lyme disease. However, there is very little objective evidence to support or disprove this hypothesis. The possibility of chronic human infection was suggested, without evidence of infection other than serology (Nicolescu and Andreescu, 1984). A single case of late-onset meningitis following icteric leptospirosis has been described (Murgatroyd, 1937), in which leptospires were isolated from both cerebrospinal fluid (CSF) and urine. This patient exhibited a negligible antibody response to the infecting strain, suggesting the presence of an immune defect.

Of the sequelae of acute leptospirosis, uveitis is a potentially chronic condition and is a recognized chronic sequel of leptospirosis in humans and horses. Equine recurrent uveitis appears to be an autoimmune disease (Lucchesi and Parma, 1999), and Faine, (1994) suggested that late-onset uveitis in humans may result from an autoimmune reaction to

subsequent exposure. Immune involvement in retinal pathology has been demonstrated in horses with spontaneous uveitis (Kalsow and Dwyer, 1998). Leptospires have been isolated from the human eye (Alexander *et al.*, 1952), and more recently, leptospiral DNA has been amplified from aqueous humor (Merien *et al.*, 1993; Chu *et al.*, 1998; Mancel *et al.*, 1999) of patients with uveitis. In these cases, uveitis has occurred relatively soon after the acute illness.

2.5. PATHOLOGY

Leptospirosis is characterized by the development of vasculitis, endothelial damage, and inflammatory infiltrates composed of monocytic cells, plasma cells, histiocytes, and neutrophils. On gross examination, petechial hemorrhages are common and may be extensive (Arean, 1962), and organs are often discolored due to the degree of icterus (Pierce, *et al.*, 1997). The histopathology is most marked in the liver, kidneys, heart, and lungs (Zaki and Spiegel, 1998), but other organs may also be affected according to the severity of the individual infection. The overall structure of the liver is not significantly disrupted, but there may be intrahepatic cholestasis (Arean, 1962; Dooley and Ishak, 1976). Hypertrophy and hyperplasia of Kupffer cells is evident (de Brito *et al.*, 1967), and erythrophagocytosis has been reported (Arean, 1962; Dooley and Ishak, 1976).

In the kidneys, interstitial nephritis is the major finding, accompanied by an intense cellular infiltration composed of neutrophils and monocytes (Penna, *et al.*, 1963). Leptospires can be seen within the renal tubules (Zaki and Spiegel, 1998). By electron microscopy, the tubular cell brush borders are denuded, the tubular basement membrane is thickened, and tubular cells exhibit mitochondrial depletion (de Brito *et al.*, 1967). In addition, minor changes are seen in the glomeruli, suggesting an anatomical basis for proteinuria in leptospirosis.

Pathological findings in the heart include interstitial myocarditis with infiltration of predominantly lymphocytes and plasma cells, petechial hemorrhages (particularly in the epicardium), mononuclear infiltration in the epicardium, pericardial effusions, and coronary arteritis (de Brito *et al.*, 1987).

In the lungs, pulmonary congestion and hemorrhage are common (Zaki *et al.*, 1996), and infiltration of alveolar spaces by monocytes and neutrophils occurs. Hyaline membrane formation may occur (Zaltzman *et al.*, 1981). Leptospires may be seen within endothelial cells in interalveolar septa, and attached to capillary endothelial cells (Nicodemo *et al.*, 1997).

In skeletal muscles, particularly of the leg, focal necrosis of isolated muscle fibers occurs, with infiltration of histiocytes, neutrophils, and plasma cells (Amato Neto and Duarte, 1992). This evidence of myositis correlates with the intense myalgia reported by some patients (Kelley, 1998). In brain, perivascular cuffing is observed (Zaki and Spiegel, 1998).

2.6. PATHOGENESIS

The mechanisms by which leptospires cause disease are not well understood. A number of putative virulence factors have been suggested, but with few exceptions their role in pathogenesis remains unclear. These are reviewed briefly below, with an emphasis on recent developments.

2.6.1. Toxin Production

The production of toxins by pathogenic leptospires in vivo was inferred by Arean, 1964. Endotoxic activity has been reported in several serovars (Isogai *et al.*, 1986; de Souza and Koury, 1992; Martinez *et al.*, 1998). Leptospiral LPS preparations exhibit activity in biological assays for endotoxin, but at much lower potencies (Masuzawa *et al.*, 1990; de Souza and Koury, 1992).

Serovar pomona is notable for the production of hemolytic disease in cattle, while serovar ballum produces similar symptoms in hamsters. Hemolysins from several serovars have been characterized. The hemolysins of serovars ballum, hardjo, pomona, and tarassovi are sphingomyelinases. Virulent strains exhibit chemotaxis towards hemoglobin (Yuri *et al.*, 1993). Plasma has been shown to prevent hemolysis (Thiermann *et al.*, 1985). Phospholipase C activity has been reported in serovar canicola. A hemolysin from serovar lai is not associated with sphingomyelinase or phospholipase activity and is thought to be a pore-forming protein (Lee *et al.*, 2000).

Strains of serovars pomona and copenhageni elaborate a protein cytotoxin and cytotoxic activity has been detected in the plasma of infected animals (Knight, *et al.*, 1973). In vivo, this toxin elicited a typical histopathologic effect, with infiltration of macrophages and polymorphonuclear cells (Yam, *et al.*, 1970). A glycolipoprotein fraction with cytotoxic activity was recovered from serovar Copenhageni. A similar fraction from sero-var canicola inhibits Na^+, K^+ ATPase (Younes-Ibrahim *et al.*, 1995). Inhibitory activity was associated with unsaturated fatty acids, particularly palmitic and oleic acids (Burth *et al.*, 1997). However, equal activity was demonstrated in *L. biflexa* serovar patoc (Burth *et al.*, 1997), implying that other virulence factors might be of greater significance.

2.6.2. Attachment

Leptospires have been shown to attach to epithelial cells. Virulent leptospires adhere to renal epithelial cells in vitro, and adhesion is enhanced by subagglutinating concentrations of homologous antibody (Ballard *et al.*, 1986). Leptospires are phagocytosed by macrophages (Pereira *et al.*, 1998) in the presence of specific antibody (Banfi *et al.*, 1982; Ballard *et al.*, 1986). Inhibition of macrophage activity increased sensitivity to infection (Isogai *et al.*, 1986). Virulent leptospires become associated with neutrophils, but are not killed. Phagocytosis occurs only in the presence of serum and complement, suggesting that the outer envelope of leptospires possesses an antiphagocytic component. Leptospiral LPS stimulated adherence of neutrophils to endothelial cells (Isogai *et al.*, 1997) and platelets, causing aggregation and suggesting a role in the development of thrombocytopenia (Isogai *et al.*, 1997).

2.6.3. Immune Mechanisms

The second stage of acute leptospirosis is also referred to as the immune phase, in which the disappearance of the organism from the bloodstream coincides with the appearance of antibodies. The clinical severity of the disease often appears to be out of proportion to the histopathological findings. Immune-mediated disease has been proposed as one factor influencing the severity of the symptoms.

The production of immune complexes leading to inflammation in the central nervous system has been postulated (Tong *et al.*, 1971). Levels of circulating immune complexes were correlated with severity of symptoms (Feresu *et al.*, 1999), and in patients who survived, circulating immune complex levels fell concurrently with clinical improvement. However, in experimental infections in guinea pigs, leptospiral antigen localized in the kidney interstitium, while immunoglobulin G (IgG) and C3 were deposited in the glomeruli and in the walls of small blood vessels (Yasuda, 1986).

The pathogenesis of equine recurrent uveitis appears to involve the production of antibodies against a leptospiral antigen which cross-react with ocular tissues (Lucchesi and Parma, 1999). Retinal damage in horses with uveitis is related to the presence of B lymphocytes in the retina (Kalsow and Dwyer, 1998). Antiplatelet antibodies have been demonstrated in human leptospirosis (Davenport *et al.*, 1989). In leptospirosis and septicemia, such antibodies are directed against cryptantigens exposed on damaged platelets and do not play a causal role in the development of thrombocytopenia (Vand der Lelie *et al.*, 1984). Other autoantibodies have been detected in acute illness, including IgG anticardiolipin antibodies (Rugman *et al.*, 1991) and antineutrophil cytoplasmic antibodies (Constantin *et al.*, 1996).

However, the significance of antineutrophil cytoplasmic antibodies in the pathogenesis of vascular injury in leptospirosis has been questioned (Abdulkader *et al.*, 1993). Virulent leptospires induce apoptosis in vivo and in vitro (Merien *et al.*, 1997 and Merien *et al.*, 1998). In mice, apoptosis of lymphocytes is elicited by LPS via induction of tumor necrosis factor alpha (TNF-α) (Isogai *et al.*, 1998). Elevated levels of inflammatory cytokines such as TNF-α have been reported in patients with leptospirosis (Estavoyer *et al.*, 1991).

2.6.4. Surface Proteins

The outer membrane of leptospires contains LPS and several lipoproteins (outer membrane proteins [OMPs]) (Haake, 2000). The LPS is highly immunogenic and is responsible for serovar specificity (de la Pena-Moctezuma, 1999). An inverse relationship between expression of transmembrane OMPs and virulence was demonstrated in serovar grippotyphosa (Haake *et al.*, 1991). Outer membrane lipoprotein LipL36 is downregulated in vivo (Barnett *et al.*, 1999) and is not recognized by the humoral immune response to host-adapted leptospirosis in hamsters (Haake *et al.*, 1998). Other OMPs are also downregulated in

vivo (Nicholson and Prescott, 1993). Outer membrane components may be important in the pathogenesis of interstitial nephritis (Barnett *et al*., 1999; Haake *et al*., 2000). A fibronectin-binding protein produced only by virulent strains was described recently (Merien *et al*., 2000).

2.6.5. Immunity to leptospiral infection

Immunity to leptospirosis is largely humoral in nature (Adler and Faine, 1977). Passive immunity can be conferred by antibodies alone (Jost *et al*., 1986; Schoone *et al*., 1989). A serovar-specific antigen (F4) extracted from LPS (Faine, *et al.*, 1974) lacked endotoxic activity and induced protective immunity in rabbits, guinea pigs, and mice (Faine, *et al.*, 1974). A similar antigen (TM), which inhibited agglutination by homologous antisera was shown to be distinct from F4 but had a common epitope (Adler and Faine, 1983). Sodium dodecyl sulfate extracts of whole cells induced production of protective antibody, which was also agglutinating and complement fixing (Kida *et al.*, 1976). Immunity is strongly restricted to the homologous serovar or closely related serovars. Serovar specificity is conferred by the LPS antigens (Vinh *et al.*, 1986b). Broadly reactive genus-specific antigens have also been described (Adler and Faine, 1983; Sonrier *et al.*, 2000).

Several of the leptospiral OMPs are highly conserved (Shang, et al., 1996; Haake *et al.*, 2000), and the potential for subunit vaccines which can generate broadly cross-protective immunity has been suggested by recent studies using OmpL1 and LipL41 (Haake *et al.*, 1999), which induced synergistic protection. Cell-mediated immune responses to leptospirosis have been reported (Ratnam *et al.*, 1984). However, suppression of the cell-mediated immune response has been reported (Yamashiro-Kanashiro *et al*., 1991), with reduction in the number of $CD4^+$ lymphocytes and in their responsiveness to some mitogens.

2.7. LABORATORY DIAGNOSIS

2.7.1 General Clinical Laboratory Findings

In anicteric disease, the erythrocyte sedimentation rate is elevated, and white cell counts range from below normal to moderately elevated (Edwards and Domm, 1960). Liver function tests show a slight elevation in aminotransferases, bilirubin, and alkaline phosphatase in the absence of jaundice. Urinalysis shows proteinuria, pyuria, and often microscopic hematuria. Hyaline and granular casts may also be present during the first week of illness.

In severe leptospirosis, a peripheral neutrophilic leukocytosis occurs with a shift to the left, whereas in dengue, atypical lymphocytes are commonly observed. Thrombocytopenia is common and may be marked (Edwards *et al.*, 1990). Renal function impairment is indicated by raised plasma creatinine levels. The degree of azotemia varies with severity of illness (Alston and Broom, 1958). In icteric leptospirosis, liver function tests generally show a significant rise in bilirubin, with lesser increases in transaminases and marginal increases in alkaline phosphatase levels (Edwards *et al.*, 1990). The increase in bilirubin is generally out of proportion to the other liver function test values (Edwards *et al.*, 1986). Similar findings were reported for serum creatinine phophokinase levels. Serum amylase may also be elevated, particularly in patients with ARF.

The nonspecific nature of these changes can only suggest a diagnosis of leptospirosis. For confirmation of the diagnosis, specific microbiological tests are necessary.

2.7.2. Microscopic Demonstration

Leptospires may be visualized in clinical material by dark-field microscopy or by immunofluorescence or light microscopy after appropriate staining. Dark-field microscopic examination of body fluids such as blood, urine, CSF, and dialysate fluid has been used but is

both insensitive and lacking specificity. Approximately 10^4 leptospires/ml are necessary for one cell per field to be visible by dark-field microscopy (Turner, 1970). A quantitative buffy coat method was recently shown to have a sensitivity of approximately 10^3 leptospires/ml (Kramer *et al.*, 1994).

A method which involved repeated microscopic examination of double-centrifuged anticoagulated blood demonstrated leptospires in 32% of patients whose leptospirosis was confirmed by animal inoculation. Microscopy of blood is of value only during the first few days of the acute illness, while leptospiremia occurs. In volunteers infected with serovar grippotyphosa, leptospires were detected as early as 4 days prior to the development of symptoms. None of the positive samples reported by Wolff (1954) were taken more than 6 days after onset of symptoms. Most authorities agree that there are too few leptospires in CSF for detection by dark-field microscopy. Direct dark-field microscopy of blood is also subject to misinterpretation of fibrin or protein threads, which may show Brownian motion (Faine *et al.*, 1999).

Staining methods have been applied to increase the sensitivity of direct microscopic examination. These have included immunofluorescence staining of bovine urine (Bolin *et al.*, 1989), water and soil and immunoperoxidase staining of blood and urine. A variety of histopathological stains have been applied to the detection of leptospires in tissues. Leptospires were first visualized by silver staining, and the Warthin-Starry stain is widely used for histologic examination. Immunofluorescence microscopy is used extensively to demonstrate leptospires in veterinary specimens (Ellis *et al.*, 1985). More recently, immunohistochemical methods have been applied (Uip *et al.*, 1992; Zaki and Spiegel, 1998; Haake *et al.*, 2000).

2.7.3. Antigen Detection

Detection of leptospiral antigens in clinical material would offer greater specificity than darkfield microscopy while having the potential for greater sensitivity. An evaluation of several methods concluded that radioimmunoassay (RIA) could detect 10^4 to 10^5 leptospires/ml and an enzyme-linked immunosorbent assay (ELISA) method could detect 10^5 leptospires/ml, but countercurrent immunoelectrophoresis and staphylococcal coagglutination were much less sensitive (Adler *et al.*, 1982). RIA was more sensitive than dark-field microscopy but less sensitive than culture when applied to porcine urine. A double-sandwich ELISA could detect 10^4 leptospires/ml of serovar hardjo but was less sensitive for other serovars (Champagne *et al.*, 1991). A chemiluminescent immunoassay was applied to human blood and urine (Palmer and Hookey, 1992) but was no more sensitive than earlier ELISA. More recently, immunomagnetic antigen capture was combined with fluoroimmunoassay to detect as few as 10^2 leptospires/ml in urine of cattle infected with serovar hardjo (Yan *et al.*, 1998). Inhibitory substances have been reported in urine (Yan *et al.*, 1998), indicating the need for treatment of urine prior to testing.

2.7.4. Isolation of Leptospires

Leptospiremia occurs during the first stage of the disease, beginning before the onset of symptoms, and has usually finished by the end of the first week of the acute illness. Therefore, blood cultures should be taken as soon as possible after the patient's presentation. One or two drops of blood are inoculated into 10 ml of semisolid medium containing 5-fluorouracil. For the greatest recovery rate, multiple cultures should be performed, but this is rarely possible. Inoculation of media with dilutions of blood samples may increase recovery (Sulzer and Jones, 1978). Rapid detection of leptospires by radiometric methods has been described (Manca *et al.*, 1986). Leptospires survive in conventional blood culture media for a

number of days. Rarely, leptospires have been isolated from blood weeks after the onset of symptoms (Jackson, 1993).

Other samples that may be cultured during the first week of illness include CSF and dialysate. Urine can be cultured from the beginning of the second week of symptomatic illness. The duration of urinary excretion varies but may last for several weeks (Bal *et al.*, 1994). Survival of leptospires in voided human urine is limited, so urine should be processed immediately (Turner, 1970) by centrifugation, followed by resuspending the sediment in phosphate-buffered saline (to neutralize the pH) and inoculating into semisolid medium containing 5-fluorouracil. Cultures are incubated at 28 to 30°C and examined weekly by darkfield microscopy for up to 13 weeks before being discarded. Contaminated cultures may be passed through a 0.2mm or 0.45mm filter before subculture into fresh medium (Rittenberg *et al.*, 1958).

2.7.4.1. Identification of leptospiral isolates

Isolated leptospires are identified either by serological methods or, more recently, by molecular techniques. Traditional methods relied on cross-agglutinin absorption (Dikken and Kmety, 1978). The numbers of laboratories which can perform these identification methods are very small. The use of panels of monoclonal antibodies (Shinozaki *et al.*, 1992) allows laboratories which can perform the microscopic agglutination test to identify isolates with relative rapidity.

2.7.4.2. Susceptibility testing

Leptospires are susceptible to β-lactams, macrolides, tetracyclines, fluoroquinolones, and streptomycin (Faine *et al.*, 1999). MBCs are several orders of magnitude higher than MICs (Takashima *et al.*, 1993). Problems in the determination of susceptibility include the

long incubation time required (Ellinghausen, 1983), the use of media containing serum (Oie *et al.*, 1983), and the difficulty in quantifying growth accurately. These constraints have limited the development of rapid, standardized methods for susceptibility testing.

2.7.5. Serological Diagnosis

Most cases of leptospirosis are diagnosed by serology. Antibodies are detectable in the blood approximately 5 to 7 days after the onset of symptoms. Serological methods can be divided into two groups:

1) Genus specific which include: Complement fixation test; sensitized erythrocyte lysis; macroscopic slide agglutination; immunfluorescence; Patoc slide agglutination test; indirect hemagglutination; counterimmunoelectrophoresis; ELISA; microcapsule agglutination; Dot-ELISA; IgM dipstick; Latex agglutination.

2) Serogroup specific.

The definitive serological investigation in leptospirosis is the microscopic agglutination test (MAT).

2.7.5.1 Microscopic agglutination test

The reference method for serological diagnosis of leptospirosis is the MAT, in which patient sera are reacted with live antigen suspensions of leptospiral serovars. After incubation, the serum-antigen mixtures are examined microscopically for agglutination, and the titers are determined. Formerly, the method was known as the agglutinationlysis test because of the formation of lysis balls or lysis globules (Van Thiel, 1948) of cellular debris in the presence of high titered antiserum. However, these are tightly agglutinated clumps of leptospires containing live cells and not debris (Turner, 1968).

Protocols for performing the MAT have been described in detail (Kaufmann and Weyant, 1995). The MAT is a complex test to control, perform, and interpret. Live cultures of all serovars required for use as antigens must be maintained. This applies equally whether the test is performed with live or formalin-killed antigens. The repeated weekly subculture of large numbers of strains presents hazards for laboratory workers, and laboratory-acquired infections have been reported (Pike, 1976). Other drawbacks include the continuous risk of cross-contamination of the antigen cultures, necessitating periodic verification of each serovar. MAT titers are affected by the culture medium in which the antigens are grown (Mayers, 1976).

The range of antigens used should include serovars representative of all serogroups and all locally common serovars (Levett, 2001). Antibody titers to local isolates are often higher than titers to laboratory stock strains of serovars within the same serogroup. It is usual to include one of the serovars of the nonpathogenic species *L. biflexa*. Such a wide range of antigens is used in order to detect infections with uncommon or previously undetected serovars (Levett, 2001). Contrary to a widely held belief, the MAT is a serogroup-specific assay. In many reports which purport to show serovar specificity, a limited range of serogroups were tested, each represented by only a single serovar. Moreover, few studies have attempted to correlate the presumptive serogroup determined by MAT with the results of culture. However, the ability of convalescent-phase MAT titers to predict even the infecting serogroup may be as low as 40% (Levett, 1999).

The MAT is read by darkfield microscopy. The end point is the highest dilution of serum at which 50% agglutination occurs. Because of the difficulty in detecting when 50% of the leptospires are agglutinated, the end point is determined by the presence of approximately 50% free, unagglutinated leptospires compared to the control suspension (Faine, 1982).

Considerable effort is required to reduce the subjective effect of observer variation, even within laboratories.

Interpretation of the MAT is complicated by the high degree of cross-reaction that occurs between different serogroups, especially in acute-phase samples. This is to some extent predictable, and patients often have similar titers to all serovars of an individual serogroup. Of note, "paradoxical" reactions in which the highest titers are detected to a serogroup unrelated to the infecting one, are also common (Brandao *et al*., 1998). The broad cross-reactivity in the acute phase, followed by relative serogroup specificity in convalescent-phase samples, results from the detection in the MAT of both IgM and IgG antibodies and the presence of several common antigens among leptospires (Lin *et al*., 1997).

Paired sera are required to confirm a diagnosis with certainty. A fourfold or greater rise in titer between paired sera confirms the diagnosis regardless of the interval between samples. The interval between the first and second samples greatly depends on the delay between onset of symptoms and presentation of the patient. If symptoms of overt leptospirosis are present, an interval of 3 to 5 days may be adequate to detect rising titers. However, if the patient presents earlier in the course of the disease or if the date of onset is not known precisely, then an interval of 10 to 14 days between samples is more appropriate. Less often, seroconversion does not occur with such rapidity, and a longer interval between samples (or repeated sampling) is necessary. MAT serology is insensitive, particularly in early acute-phase specimens (Cumberland *et al.*, 1999). Moreover, patients with fulminant leptospirosis may die before seroconversion occurs (Cumberland *et al.*, 1999).

Titers following acute infection may be extremely high (≥25,600) and may take months or even years to fall to low levels (Lupidi *et al.*, 1991). Often, it is not possible to distinguish a predominant serogroup until months after infection, as cross-reacting titers decline at different rates (Lupidi *et al.*, 1991). If possible, it is important to examine several

sera taken at intervals after the acute disease in order to determine the presumptive infecting serogroup. Rarely, seroconversion may be delayed for many weeks after recovery, and longer serological follow-up will be necessary to confirm the diagnosis.

Formalized antigens have been used in the MAT to overcome some of the difficulties associated with the use of live antigens. Titers obtained with these antigens are somewhat lower, and more cross-reactions are detected (Levett, 2001). Agglutination of formalin-treated antigens is qualitatively different from that seen with live antigens (Alexander, 1986); however, for laboratories without the staff or expertise to maintain live antigens, formalin-treated and lyophilized antigens may represent a good alternative.

The MAT is also the most appropriate test to employ in epidemiological serosurveys, since it can be applied to sera from any animal species and the range of antigens used can be expanded or decreased as required. It is usual to use a titer of ≥100 as evidence of past exposure (Faine, 1982). However, conclusions about infecting serovars cannot be drawn without isolates at best; the MAT data can give a general impression about which serogroups are present within a population.

2.7.5.2. Other serological tests

Because of the complexity of the MAT, rapid screening tests for leptospiral antibodies in acute infection have been developed. Complement fixation (CF) was applied to veterinary diagnosis, but species-specific differences were noted (Robertson, 1963). CF tests have generally been replaced by ELISA methods (Levett, 2001). IgM antibodies become detectable during the first week of illness (Levett, 2001), allowing the diagnosis to be confirmed and treatment initiated while it is likely to be most effective.

IgM detection has repeatedly been shown to be more sensitive than MAT when the first specimen is taken early in the acute phase of the illness (Cumberland *et al.*, 1999). IgM

antibodies have been detected by ELISA in CSF from patients with icteric leptospirosis. In patients with meningitis without a proven etiology, IgM was detected in the CSF in 15% (Silva *et al.*, 1996). IgM has been detected in saliva (Silva *et al.*, 1997), and a dot-ELISA using polyester fiber was developed to facilitate collection of saliva directly onto the support material (Silva *et al.*, 1994).

ELISA methods have been applied in a number of modifications. An IgM-specific dot-ELISA was developed in which polyvalent leptospiral antigen was dotted onto nitrocellulose filter disks in microtiter tray wells, allowing the use of smaller volumes of reagents. Further modifications of this approach have been used to detect IgG and IgA in addition to IgM (Silva *et al.*, 1997) and have employed an immunodominant antigen (Ribeiro *et al.*, 1995) and a polyester fabric-resin support in place of nitrocellulose (Silva *et al.*, 1994).

A commercial IgM dot-ELISA dipstick has been shown to be as sensitive as a micro titer plate IgM-ELISA (Levett *et al.*, 2001). Another dipstick assay (Silva *et al.*, 1994) has been extensively evaluated in several populations (Sehgal *et al.*, 1999). A dot immunoblot assay using colloidal gold conjugate allowed completion of the assay within 30 min (Petchclai *et al.*, 1991). In contrast to the applications of ELISA for diagnosis of human infection, in which broadly reactive assays are generally desirable and few serovar-specific assays have been developed (Milner *et al.*, 1985), veterinary applications have been directed towards detection of serovar-specific antibodies, particularly for detection of infection in food animals. ELISA methods have described for detection of serovar pomona (Cousins *et al.*, 1985) and hardjo (Yan *et al.*, 1999) infection in cattle and hardjo in sheep. Several assays are available commercially for serodiagnosis of bovine hardjo infection and have been evaluated (Woodward *et al.*, 1997). IgM detection by ELISA has also been applied to canine diagnosis (Weekes *et al.*, 1997).

A macroscopic slide agglutination test was described in which 12 serovars were combined into four pools for the rapid screening of sera from humans and animals. Despite the use of an expanded antigen range, false-negative results were reported for sera from populations in areas of endemic leptospirosis (Wolff and Borhlander, 1966). Several modifications of this test have used a single serovar antigen, usually serovar patoc (Martin-Leon *et al.*, 1997). Some studies have reported that the patoc slide test is insensitive (Martin-Leon *et al.*, 1997), but a commercial slide agglutination assay was recently found to be as sensitive and specific as an IgM-ELISA while remaining reactive for a shorter time after recovery than either the IgM-ELISA or the MAT (Brandao *et al.*, 1998).

A number of methods using sensitized red blood cells have been described. The extraction of an erythrocyte-sensitizing substance led to the development of both a hemolytic assay requiring complement (Cox *et al.*, 1957) and a hemagglutination assay (Sulzer and Jones, 1973), and a number of modifications of the latter have been described (Sakamoto *et al.*, 1985). These assays detect both IgM and IgG antibodies (Levett and Whittington, 1998). The indirect hemagglutination assay (IHA) developed at CDC was shown to have a sensitivity of 92% and specificity of 95% compared with the MAT (Sulzer *et al.*, 1975).

This assay is available commercially and for many years as the only U.S. Food and Drug Administration-approved product for serological diagnosis of leptospirosis. Recent estimates of the sensitivity of the IHA in populations in which leptospirosis is endemic have varied. In one study, IHA detected all patients with leptospirosis but was positive in only 44% of first acute-phase samples taken a mean of 5 days after onset of symptoms (Levett and Whittington, 1998). Other studies have reported lower overall sensitivities, partly due to differences in case ascertainment and study design (Yersin *et al.*, 1999; Effler *et al.*, 2000).

A microcapsule agglutination test using a synthetic polymer in place of red blood cells has been evaluated extensively in Japan and China (Cui *et al.*, 1991). In an international

multicenter evaluation, the microcapsule agglutination test was more sensitive than either the MAT or an IgM-ELISA in early-acute-phase samples (Arimitsu, 1994), but failed to detect infections caused by some serovars. Advantages of this direct agglutination method is that it can be applied without modification to sera from other animal species. Other techniques applied to the detection of leptospiral antibodies include immunofluorescence (Appassahij *et al.*, 1995), Radioimmunoassay (Kawaoka *et al.*, 1979), counterimmunoelectrophoresis (Yasuda *et al.*, 1991), and thin-layer immunoassay (Banfi *et al.*, 1984). These methods have not been widely used.

2.7.6. Molecular Diagnosis

Leptospiral DNA has been detected in clinical material by dot-blotting (Millar *et al.*, 1987) and in situ hybridization (Terpstra *et al.*, 1987). A recombinant probe specific for pathogenic serovars was prepared from serovar lai (da Silva *et al.*, 1990). Probes specific for serovar hardjobovis were developed (Zuerner and Bolin, 1988) and applied to the detection of leptospires in bovine urine. However, the sensitivity of ^{32}P-labeled probes was approximately 10^3 leptospires (Millar *et al.*, 1987), much lower than the sensitivity of PCR, and probes have not been used extensively for diagnosis since PCR be-came available.

Several primer pairs for PCR detection of leptospires have been described, some based on specific gene targets (Renesto *et al.*, 2000), and most frequently 16S or 23S rRNA genes (Hookey, 1992; Murien *et al.*, 1992) and repetitive elements (Pacciarini *et al.*, 1993; Savio *et al.*, 1994), while others have been constructed from genomic libraries. However, few have been shown to amplify leptospiral DNA from either human (Gravekamp *et al.*, 1991; Merien *et al.*, 1992) or veterinary (Masri *et al.*, 1997; Taylor *et al.*, 1997) clinical material, and of these, only two methods have been subjected to extensive clinical evaluation (Merien *et al.*, 1995; Brown *et al.*, 1995). Both methods were found to be more sensitive than culture,

but differences in analysis of the data render direct comparisons between the two approaches impossible.

Both of these approaches have limitations. The primers described by Merien *et al.*, (1992) amplify a 331-bp fragment of the *rrs* (16S rRNA) gene of both pathogenic and nonpathogenic leptospires, which in the unlikely event of contamination of specimens with nonpathogenic leptospires might produce a false-positive result, whereas the G1 and G2 primers described by Gravekamp *et al* (1991), do not amplify *L. kirschneri* serovars, necessitating the use of two primer pairs for detection of all pathogenic serovars (Gravekamp *et al.*, 1993). Despite these observing shortcomings, these two primer pairs have been the most widely used for clinical studies. Leptospiral DNA has been amplified from serum, urine (Bal *et al.*, 1994; Brown *et al.*, 1995), aqueous humor (Murien *et al.*, 1993) and CSF (Merien *et al.*, 1995; Vinetz *et al.*, 1996; Romero *et al.*, 1998).

The detection of leptospiral DNA in bovine urine has also been investigated. Primers which amplified several serovars of serogroup Sejroe were described (Van Eys *et al.*, 1991), and a method specific for serovar hardjo genotype hardjobovis was developed (Woodward *et al.*, 1991). An assay based on the I*S1533* insertion sequence (Zuerner *et al.*, 1995) facilitated both detection and identification of serovars directly from urine. Another assay was developed and applied to both bovine and porcine urine samples (Wagenaar *et al.*, 1994). To overcome the problem of inhibitors present in bovine urine, a magnetic immunocapature PCR assay for serovar hardjo was developed (Taylor *et al.*, 1997).

A recent study evaluated five PCR methods, culture, and immunofluorescence for detection of serovar hardjo in bovine urine samples (Wagenaar *et al.*, 2000). Primers derived from rRNA gene sequences were the least specific, and none of the methods was 100% sensitive. A combination of two detection methods chosen from PCR, immunofluorescence, and culture was the most sensitive.

A limitation of PCR-based diagnosis of leptospirosis is the inability of most PCR assays to identify the infecting serovar. While this is not significant for individual patient management, the identity of the serovar has significant epidemiological and public health value. Strategies designed to overcome this obstacle have included restriction endonuclease digestion of PCR products (Savio *et al.*, 1994; Brown and Levett, 1997), direct sequencing of amplicons (Oliveira *et al.*, 1995), and single-strand conformation analysis (SSCP) (Matthias, 2000). Leptospiral genomospecies but not individual serovars can be differentiated following PCR by electrophoresis in nondenaturing polyacrylamide gels, followed by silver staining (Oliveira *et al.*, 1995), without the additional step of purification and denaturing. PCR has been used to distinguish pathogenic from non-pathogenic serovars (Murgia *et al.*, 1997; Parma *et al.*, 1997; Woo *et al.*, 1997b). Recently, a fluorescent-probe 59 exonuclease PCR assay was described for the rapid detection of pathogenic leptospires (Woo *et al.*, 1998a).

2.7.7. Molecular Typing

Because of the difficulties associated with serological identification of leptospiral isolates, there has been great interest in molecular methods for identification and subtyping (Terpstra, 1992; Herrmann, 1993). Methods employed have included digestion of chromosomal DNA by restriction endonucleases (REA), restriction fragment length polymorphism (RFLP), ribotyping, PFGE, and a number of PCR-based approaches.

REA has been studied extensively (Terpstra *et al.*, 1987; Tamai *et al.*, 1988; Hookey and Palmer, 1991). Distinct genotypes within serovar hardjo were demonstrated (Robinson *et al.*, 1982). Bovine isolates from North America have all been found to be of genotype hardjobovis, of which subtypes A, B, and C could be recognized (Thiermann *et al.*, 1986). In Northern Ireland, both genotypes hardjobovis and hardjoprajitno were found among bovine isolates (Marshall *et al.*, 1985). Antigenic differences were also reported among hardjobovis

isolates (LeFebvre *et al.*, 1987). Moreover, serovar balcanica isolates in North America were indistinguishable from genotype hardjobovis isolates by REA (Thiermann *et al.*, 1986). Further analysis of RFLP in genotype hardjobovis isolates by REA, Southern blotting, and PFGE has shown the existence of multiple genetic clones resulting from genomic rearrangement (Zuerner *et al.*, 1993). These clones were usually localized within geographical locations and thus are of epidemiological significance (Zuerner *et al.*, 1993).

Similar subserovar differences were detected within serovar pomona, isolates from North America being identified as subtype kennewicki while European isolates were of serovar pomona or mozdok (Hathaway *et al.*, 1985a). More recently, differences between subtype kennewicki isolates were correlated with host animal source (Bolin and Zuerner, 1996). Differences between serovars copenhageni and icterohaemorrhagiae were demonstrated by some workers but not all (Hookey and Palmer, 1991). However, all isolates of these two serovars are indistinguishable by PFGE (Herrmann *et al.*, 1991).

Ribotyping has demonstrated reasonably good correlation with the phylogenetic classification of leptospira into 11 genomospecies. Using *Eco*RI for digestion and 16S and 23S rRNA from *Escherichia coli* as the probe, a large database was constructed (Perolat *et al.*, 1990; Perolat *et al.*, 1993). Many serovars gave unique profiles, while other serovars could not be distinguished from each other by ribotyping, particularly those that were known previously to be closely related, such as icterohaemorrhagiae and copenhageni (Hookey and Palmer, 1991). Ribotyping has been shown to discriminate accurately between the serovar hardjo genotypes hardjobovis and hardoprajitno (Perolat *et al.*, 1994).

An alternative approach to ribotyping used three restriction enzymes and a PCR-derived 16S rDNA probe. Use of only 16S rDNA gave fewer bands, but this was counterbalanced to some extent by the use of multiple restriction enzymes, giving three different patterns for each serovar (Hookey, 1993). Relatively few serovars were examined

but all gave distinct ribotypes with the exception of serovars icterohaemorrhagiae and copenhageni. A range of other probes have been used to generate RFLPs (Zuerner and Bolin, 1990; Van Eys *et al.*, 1991; Pacciarini *et al.*, 1992). A probe based on the repetitive sequence element from serovar hardjo genotype hardjobovis was also used to detect leptospires in bovine urine (Zuerner and Bolin, 1988).

PFGE has proven useful to characterize leptospiral serovars. In contrast to its application in strain typing of other organisms, PFGE has shown that the genomes of leptospiral serovars are remarkably conserved, both over time and across wide geographical distributions (Herrmann, 1999). Importantly, recent clinical isolates gave the same banding patterns as reference strains of the same serovar which have been maintained for many years by repeated subculture (Herrmann, 1991). Using the enzyme *Not*I, most but not all serovars gave unique PFGE patterns. *L. interrogans* serovars bratislava, lora, jalna, and muenchen gave identical patterns when digested with *Not*I but were differen-tiated when digested with *Sg*rAI (Herrmann *et al.*, 1992).

Other serovars which were difficult to differentiate included *L. borgpetersenii* serovars arborea and castellonis. The *L. interrogans* serovars copenhageni and icterohaemorrhagiae were indistinguishable by PFGE, confirming their close relationship. PFGE analysis has become the de facto standard for molecular characterization of leptospiral isolates, and other molecular typing methods will in future have to be validated against this method.

A limiting factor in all methods which analyze chromosomal DNA is the requirement for large quantities of purified DNA. As a result, several methods based on the analysis of PCR-amplified sections of leptospiral DNA have been employed. Sequence variation within the 285-bp fragment amplified by the G1 and G2 primers led to different electrophoretic mobilities which were detected by polyacrylamide gel electro-phoresis and silver staining

(Oliveira *et al.*, 1995). This approach allowed se-rovars of *L. interrogans* sensu stricto to be differentiated from *L. noguchii* serovars.

Sequence variation is also exploited in SSCP. Using this method, serovars prevalent in China were shown to have dif-ferent mobilities corresponding to *L. interrogans* and *L. borgpetersenii* (Wu *et al.*, 1996). The Chinese isolates were studied using a sequence amplified from the 16S rRNA gene, the highly con-served nature of which may account for the inability to distinguish serovars from one another. In contrast, SSCP analysis of the G1-G2 amplicon allows serovar identification within each genomospecies studied (Matthias, 2000). A restriction on the use of the latter sequence is the inability of the G1 and G2 primers to amplify *L. kirschneri* (Gravekamp *et al.*, 1993). An alternative application of these primers is their use under low-stringency conditions, generating a mixture of specific and nonspecific products. Under these conditions, the G1 and G2 primers amplify all species, including *L. biflex*a. Polymorphisms were detected which allowed discrimination of serovars with the exception of closely related serovars, including copenhageni and icterohaemorrhagiae (de Caballero, 1994; Brown *et al.*, 1995).

The presence of multiple copy insertion sequences has been exploited for serovar identification (Zuerner *et al.*, 1995; Zuerner and Bolin, 1997). Methods based on I*S1533* have limited application because of the absence of this insertion sequence in *L. interrogans* (sensu stricto) and *L. noguchii* (Zuerner *et al.*, 1995). By amplifying the sequences between adjacent copies of I*S150*0, numerous genetic sub-groups within serovar pomona type kennewickii were distinguished (Zuerner and Bolin, 1997).

RFLP analysis of PCR-amplified 16S and 23S rRNA genes allowed the grouping of 48 serovars into 16 mapped restriction site polymorphism profiles (Ralph *et al.*, 1993). Using this approach, the genomospecies of *Leptospira* could be identified, and the ge-notypes hardjobovis and hardjoprajitno of serovar hardjo were clearly distinguished (Perolat *et al.*,

1994). The method was simplified to yield only five profiles by using a single restriction enzyme (Woo *et al.*, 1997a). One of the potential advantages of this RFLP approach is the ability to amplify leptospiral DNA from clinical material and to identify the infecting serovar or genomospecies rapidly in the absence of an isolate. Other workers have used primers that amplify only a restricted range of serovars (Savio *et al.*, 1994; Brown and Levett, 1997), limiting the utility of the approach unless several primer sets are used (Brown and Levett, 1997).

DNA fingerprinting using arbitrary primers (Williams *et al.*, 1990; Welsh and McClelland, 1990) has been studied extensively (Perolat *et al.*, 1994; Brown and Levett, 1997), using different primers and conditions. Direct comparison between the results of these studies is therefore impossible, but it is clear that reproducibility is difficult to achieve without absolute standardization of experimental procedure. Profiles are affected markedly by the primer used, the quantity and quality of the DNA template (Corney *et al.*, 1993; Matthias, 2000), and the electrophoresis conditions (Corney *et al.*, 1997).

The greatest value of arbitrary primer techniques lies in their ability to differentiate between isolates when the range of potential serovars is limited, allowing rapid identification of freshly isolated strains (Corney *et al.*, 1993; Brown and Levett, 1997). Arbitrary- primed PCR was used to derive species-specific probes for identification of *L. interrogans* (sensu stricto), *L. borg-petersenii*, and *L. kirschneri* by dot blotting (Letocart *et al.*, 1997). A cluster of 43 *L. interrogans* sensu strico isolates from a number of Brazilian outbreaks were shown to have identical arbitrary-primed PCR fingerprints (Pereira *et al.*, 2000) despite the inclusion of isolates of serovars copenhageni and canicola.

CHAPTER THREE

3.0 RETROSPECTIVE AND PROSPECTIVE STUDIES ON CANINE LEPTOSPIROSIS IN SOUTHWESTERN NIGERIA

3.1. INTRODUCTION

Leptospirosis is a worldwide zoonosis caused by *Leptospira interrogans* which include all pathogenic strains of the Genus *Leptospira.* More than 200 pathogenic serovars have been identified serologically and serotyped according to their antigenic relation (Levett, 2001). Leptospirosis has been reported in different parts of the world. In many instances, serovar implicated include *L. icterohaemorrhagiae, L. canicola, L. bratislava, L. pomona, L. autumnalis* and *L. grippotyphosa.* Until recently, *L. icterohaemorrhagiae* and *L. canicola* have been associated with clinical diseases in dogs (Hartmann *et al.,* 1984; Harkin *et al.,* 1996; McDonough, 2005). The transmission of leptospire generally occurs after a susceptible animal is directly exposed or brought in contact with the infected host's urine or contaminated water, mud, moist soil (Levett, 2001; Ward *et al.,* 2004b; Andre-Fontaine, 2006).

In the reservoir host, leptospires escape the immune system in the proximal convoluted tubules allowing the infected animals to become persistent shedders (Wohl, 1995). Following shedding in urine, these bacteria may survive for some months given appropriate temperature, moist or wet environment. However, survival is very poor in the dry or cold environment (Faine, 1962; Leonard *et al*., 1993). Conflicting reports abound in the literature on the effects of risk factors such as age, sex and breed on canine leptospirosis, although the effect of the environmental factors such as increased rainfall, warmer temperature, and seasonality of leptospirosis cases have been fairly consistent (Ward, 2002; Prescott *et al.,* 2002; Ward *et al.,* 2002). After natural infection with *Leptospira* in the incidental host, the spectrum of infection in the dog ranges from acute to subacute or chronic (McDonough, 2005). The

pathogenesis of the disease involves the penetration of the mucous membrane or abraided skin and multiplies rapidly upon entering the blood. The organism further replicates in many tissues, including the kidney, liver, spleen, central nervous system, eyes and genital tract. Increase in serum antibodies thereafter clears the spirochetes from most organs, but persist in the kidney to cause persistent leptospiruria (Langston and Heuter, 2003). These clinical forms are influenced by virulence factors, the immune status of the dog and the host adaptation (i.e. primary reservoir host or incidental host) (Heath and Johnson, 1994). The disease in primary reservoir hosts tends to be more chronic or asymptomatic with weak antibody responses. In contrast, the disease in incidental hosts tends to be acute and severe with marked antibody responses (McDonough, 2005). Although, the mechanism of action by which leptospire cause disease appears to be obscure and not well understood, but toxin and enzymes had been incriminated to contribute to their pathogenicity. The common clinical signs in dogs include hypothermia, pyrexia, depression, anorexia, vomiting, abdominal pain, icterus, congestion of ocular mucosa, diarrhoea (Keenan, 1978, Harkin *et al.*, 1996, Adin and Cowgill, 2000).

Haematological alterations commonly observed in leptospirosis are intense leukocytosis due to neutrophilia and varied degree of anemia and severely affected dogs may present thrombocytopenia (Greene *et al.*, 2006).

Recent clinical observations and serological surveys in Europe and North America demonstrated that new emergent serovars particulary sejroe, grippotyphosa and bratislava were associated with leptospiral infection in dogs (Scanziani 1995, Rühl-Fehlert, 2000; Scanziani 2002, Harkin *et al.*, 2003). In Nigeria, unpublished data have shown over the years that many dogs have died of severe clinical syndrome, pathologically attributed to acute renal failure associated with leptospirosis in Department of Veterinary Pathology of the two Veterinary Schools in southwestern Nigeria. Recently, Okewole and Ayoola, (2009) serologically showed that other serovars (*L. pomona, L. bratislava, L. grippotyphosa*) apart

from *L. icterohaemorrhagiae* and *L. canicola* are possible causes of death in dogs in Southwestern part of the Nigeria. Moreover, no data are available regarding the prevalence of chronic infections by these serovars in dogs as the microscopic agglutination test (used for the serological surveys) demonstrates mainly the recent infections (Klaasen, 2003). Clinical diagnosis of leptospirosis can be challenging and multiple concurrent diagnostic methods are often used since clinical signs are often non-specific. Serological tests which include microscopic agglutination test and enzyme-linked immunosorbent assay, fluorescent antibody tests, dark field microscopy, culture isolation, polymerase chain reaction (PCR) assay and histopathology with special stains (Warthin Starry silver stain, immunohistochemistry) could be used to identify leptospires in the tissue or body fluid of dogs (Wild *et al.*, 2002).

Despite the plethora of information in the literature on canine leptospirosis, few works have been able to dwell on culture isolation and characterization of leptospiral organism with the associated pathology using different diagnostic methods such as EMJH medium, monoclonal antibodies, Warthin Starry silver stain and immunohistochemistry, as most of the studies were based on the sero-epidemiological survey in canine populations (Gautam *et al.*, 2010). Moreover, since leptospiral organism possess the ability to change host specificity and virulence in response to selective pressure in the environment (Greene and Shotts, 1990), it is pertinent that studies on the prevalent serovars of leptospira should be undertaken in order to determine whether there are new leptospira serovars in the dog population in Nigeria.

Therefore, the objectives of this study were 1) to determine the prevalence of canine of leptospirosis between 2003 to 2010 in the Southwestern zone of Nigeria, 2) to determine the effects of risk factors such as age, breed and sex on prevalence of canine leptospirosis, 3) to characterize the prevailing serovars in dogs, 4) to determine the clinico-haematological and biochemical changes in naturally infected dogs, 5) to determine the associated pathology of canine leptospirosis in naturally infected dogs using modern techniuques.

3.2 MATERIALS AND METHODS

3.2.1 Data Source

Retrospective examination of the Veterinary records database of dogs received at the Veterinary Teaching Hospital of the two Veterinary schools in southwestern Nigeria from 2003 to 2010 were carried out. Information on age at diagnosis, sex, breed and date of diagnosis was extracted from the files of these two Veterinary Teaching Hospitals. Haematological and biochemical profiles of these dogs were also examined. Postmortem cases of dogs submitted to the two Departments of Veterinary Pathology of the two Veterinary schools were also used for the prospective study of cases of suspected canine leptospirosis.

3.2.2 Signalment

All the cases of dogs in this study (between 2003-2010) were analyzed for any difference in sex, breed and age. Five hundred and seventeen dogs were used to determine the influence of sex on the occurrence of leptospirosis. Sex was categorized as male or female and those in which their sex were not specified in the record. Breeds of dogs were classified as exotic, local, mixed breeds or undetermined based on the records. Five hundred and four cases were classified according to breed. Dogs were categorized into four age groups, viz: <1 year, 1- 4 years, >4years and those in which their age were not specified.

3.2.3 Clinical and pathological findings in dogs suspected of leptospirosis

Clinical and clinicopathological data of dogs admitted between 2003 and 2010 to the Veterinary Teaching Hospital with suspected and non-suspected cases of leptospirosis were analyzed. Criteria for diagnosis of canine leptospirosis with no alternative diagnosis such as history of anorexia and jaundice with absence of ticks on the body, clinical signs on presentation were examined in 116 cases. Abnormal and normal parameters of

haematological importance such as packed cell volume (PCV), red blood cell (RBC), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), white blood cell count (WBC), platelets count and WBC differentials were determined by standard methods in 279 cases. Biochemical parameters such as blood urea nitrogen (BUN), creatinine, alkaline phosphatase (ALP), alanine aminotransferase (ALT), and bilirubin were also determined in 7 dogs using Randox test kits.

3.2.4 Selection and exclusion criteria

Dogs were selected or suspected of leptospirosis when clinical signs such as jaundice, anorexia, vomiting, pyrexia and absence of ticks on the body were evidenced at presentation. Dogs were also suspected of other disease condition such as babesiosis, trypanosomiasis, and erhlichiosis when the organisms are present in the thin blood-smear stained with Giemsa stain. Dogs brought to the two Veterinary Teaching Hospitals for routine examination and vaccination without any sign of disease were regarded as normal. Cases such as tumor or accident or cases associated with poisoning were excluded.

3.2.5 Culture Isolation from blood and kidney of dogs suspected of leptospirosis

One to 2 drops of blood sample were inoculated into 5ml of EMJH medium and 10ml of the medium was added to a final volume of 15ml. This was again divided into 3 to make 5ml in each bottle. After 24hrs, these samples were examined under dark field microscopy for the presence of spiral-shaped organisms.

Kidneys of 41 dogs suspected of leptospirosis from necropsy were collected over a period of two years and rinsed with sterile distilled water and put into sterile Petri dishes. A small piece of 0.5 cm^3, containing the cortex and medulla was cut and macerated using sterile rat toothed forceps in the Petri-dish and then suspended in 0.5 ml PBS (pH 8.0). The suspension was then allowed to stay for 10 minutes at room temperature to allow the leptospire organisms to flow out from the macerated kidney into the PBS.

Two to three drops of the kidney suspensions were inoculated into 5ml of EMJH-Florouracil medium. This was incubated at room temperature (28-30ºC) in the dark and examined under dark field illumination within 24 hours and at intervals of 10 days to check for the growth of leptospires for at least three months.

3.2.6 DNA Extraction

Genomic DNA was extracted from seven days old isolates grown at 30º C in EMJH medium and harvested by centrifugation at 1000g for 10 minutes. The pellet was washed twice and dissolved in 500 µL solution I (10 mM Tris, 10 mM of $MgCl_2$, 2 mM of ethylenediaminetetra acetic acid (EDTA), 400 mM NaCl and 10 mM KCl in 500 ml of millipore water). 50 µL of lysozyme (5mg/mL dissolved in solution I) was added, followed by incubation at 37ºC for 15 mins. Then 50 µL of 10 % SDS and 5µL Proteinase K (10 mg/mL) were added and incubated at 65ºC for 30 mins. 40 µL of 5 M NaCl and 32 µL of CTAB were added and again incubated at 65ºC for 30 mins. Then equal volume of chloroform and isoamylalcohol (24:1) were added. The content was vortexed and centrifuged at 10,000 g for 10 mins. The supernatant was mixed with 0.6 % volume of chilled ethanol and kept in -70ºC for one hour. The vials were spun at 12,000 g for 30 mins and the pellet was air dried, dissolved in 50 µL of Tris-EDTA (TE) buffer and stored at -20ºC until it was used. The primers used were G1 (5'-CTGAATCGCTGTATAAAAGT-3') G2 (5'-GGAAAACA AATGGTCGGAAG-3'). These primers are traditionally known to be diagnostic of leptospirosis and were described by Gravekamp *et al.* (1993). PCR amplification was carried out in a total volume of 25µL,

Amplification was processed in a thermocycler with an initial cycle of 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 51°C for 1 min and 72°C for 1 min, with a final extension of 72°C for 7 min. The PCR product was analyzed by electrophoresis in 1.5% agarose gel, stained with ethidium bromide (0.5 mg/ml) and visualized by UV trans-illuminator

3.2.7 Characterization of leptospiral isolates from kidney and blood of infected dogs

Microscopic Agglutination test (MAT) was performed with the monoclonal antibodies obtained from the Royal Tropical Institute, Netherland (WHO/FAO/OIE and National Collaborating Centre for Reference and Research on Leptospirosis) using six leptospiral serovars as antibodies. The serovars belong to the serogroups, Canicola (Hond Utrecht IV), Icterohaemorrhagiae (RGA), Grippotyphosa (Moskova V), Pomona (Pomona), Bratislava (Jez Bratislava), and Hardjo (Hardjoprajitno). The characterization of 27 pure leptospiral isolates in this study was performed according to the method of Obregón *et al.*, (2007). Briefly, monoclonal antibodies (mAbs) were prepared at the ratio of 1: 100 (i.e. 1µl of mAb was added to 100 µl of PBS). To each of the 2nd to 6th well in a row of the microplate was added 50 µl of PBS. This was followed by addition of 50 µl of mAbs to only wells 1 and 2. Thus, each A, B, C, D, E, and F rows corresponded to each of the mAbs which include A= *L. Pomona*, B= *L. Grippotyphosa*, C= *L. Hardjo*, D= *L. Bratislava*, E= *L. Canicola* and F= *L. icterohaemorrhagiae*.

Serial dilution from well 2 to 6 was performed and 50µl of live 7 day old leptospiral isolate was added to each of the well 1 to 6. The plates were covered with plate sealers and incubated at 37^{0}C for 2hrs. After incubation, a drop (5 µl) of mixture of final dilution from 100, 200, 400, 800, 1600 and 3200 was placed on grease free slide and covered with cover-slip.This was viewed using 40X objective of the dark-field microscope (Leica, U.K) for the presence of agglutination and/ or reduction in the number of organism. A 50% reduction in the number of free leptospires in the test sample was considered positive with or without agglutination. The highest dilution which showed 50% reduction in the number of free leptospires with or without agglutination was recorded as the respective titre.

3.2.8 Formalinized organ sections from Archives.

Formalinized tissues such as kidney liver, spleen, lymph nodes and pancreas of sixty cases of suspected leptospirosis between 2003 - 2010 were retrieved from the archives of the Departments of Veterinary Pathology, University of Ibadan and University of Agriculture, Abeokuta Nigeria. Selection was based on clinical history of acute febrile disease, morphologic diagnosis of renal haemorrhages, tubular nephrosis, chronic interstitial nephritis, and documented or suspected cases of leptospirosis. The working definition of a suspected case of leptospirosis in this study was a dog with clinical signs, clinicopathological and gross morphological changes such as absence of ticks on the body, jaundice, bleeding diathesis in different organs and histological nephrosis with interstitial nephritis.

3.2.9 Histopathological examination of organs of Leptospiral infected dogs

Organ sections such as liver, kidney, spleen, lymph nodes, pancreas, brain and adrenal gland were histopathologically evaluated. They were collected into neutral-buffered 10% formalin and processed via standard paraffin-embedding techniques. Sections were cut at 5 µm. All sections were stained with H&E. Histopathological grading of selected lesions was carried out according to the grading of Greenlee *et al*, (2004). The liver, spleen, lymph nodes, pancreas and kidney lesions were graded using mild (+), moderate (++), and severe (+++) designations depending on the degree of the change. For interstitial nephritis, tubular mineralization, and hepatic perivascular inflammation, the scores were assigned as follows: (–) = no change, (+) = 1 to 3 foci/section examined, (++) = 4 to 6 foci/section examined, (+++) = > 6 foci/section examined. Tubular degeneration and necrosis was graded (+) when scattered cells were detected with pyknotic, karyorrhexis, karyolysis nuclei or loss of polarity, (++) when these changes were present in larger sections of a tubule, and (+++) when multiple tubules in an area were affected. Tubular dilation was scored (+) when scattered

tubular lumens were severely widened and lined by a thin layer of epithelium, (++) when multiple tubules were affected, and (+++) when the cortex was diffusely affected.

Selected sections were also stained with Warthin starry silver (WSS) stain (appendix 1) and Periodic Acid Schiff (PAS) stain.

These cases were divided into 2 groups: group 1 included dog in which leptospires were detected by silver staining of tissue, and group 2 included dogs in which leptospires or leptospiral fragments were not observed in tissue sections. Various renal lesions were recorded from both groups and the lesions were numerically graded according to the numbers of different histopathological changes in the tissues of each group.

3.2.10 Immunohistochemical staining of kidney and liver in dogs suspected of leptospirosis

Serial 5-µm sections from each paraffin block were mounted on slides and allowed to dry overnight. The sections were deparaffinized in 4 changes of xylene for 3 min each, rehydrated in a graded series of ethanol solutions (100%, 95%, 70%, 50%), and finally washed with deionized water. The deparaffinized sections were then placed in a Coplin jar containing 0.01M sodium citrate buffer (pH 6.0) and heated for 40-mins periods, followed by cooling to room temperature for 20 mins, to unmask the antigens. Following 5 min of washing in 0.05M phosphate-buffered saline (PBS) solution (pH 7.6) containing 0.05% Tween 20 (TPBS). All steps were done at room temperature. The sections were first incubated in 3% hydrogen peroxide for 15 min to quench endogenous peroxidase.

After a brief wash of TPBS, nonspecific binding was blocked by bathing in normal goat serum (1:10 dilution in PBS) for 10 min. The tissues were then incubated with specific monoclonal antibody (1:800 dilutions in PBS) for 30 min. After a brief wash with TPBS, the slides were incubated with biotinylated secondary antibody (goat anti-rabbit IgG) for 15 min.

After a last wash in TPBS, the slides were incubated with streptavidin– biotin–horseradish peroxidase for 15 min. Slides were then rinsed with distilled water and incubated with the chromogen 3-amino- 9-ethylcarbazole-peroxidase for 10 mins. After a final rinse with distilled water to stop the staining process, slides were removed from the autostainer.

Sections were counterstained with Mayer hematoxylin for 3 min followed by a 5-min rinse in running tap water and were mounted with glycerol water-soluble mounting medium for microscopic evaluation.

3.2.11 Determination of both acute and chronic infection in canine leptospirosis using Warthin Starry silver stain and immunohistochemistry

From the kidney samples that were positive to leptospiral infection, different stages or distribution of leptospiral organism within the renal parenchyma were used to determine whether leptospiral infections in individual dogs were acute or chronic according to the studies of different workers (Yener and Keles, 2001; Tuncuduva *et al.*, 2007; Cherille *et al.*, 1980; Sitpirija *et al*., 1980; Marshall, 1980)

The determinations were based on the following assumptions:

3.2.11.1 Observation /criteria for acute infection

(1) Presence of leptospiral organisms within the interstitium (Cherille *et al.*, 1980; Sitpirija *et al*., 1980).

(2) Presence of leptospires in the protein hyaline cast especially within the Bowman's spaces and the proximal convoluted tubules (Cherille *et al.*, 1980; Sitpirija *et al.*, 1980).

(3) Observation of leptospire strands which spanned between the interstitium via the tubular basement membrane into the cytoplasm of the tubular epithelial cells (Sitpirija *et al*., 1980).

(4) The presence of a whole leptospire organism within the cytoplasm of the tubular epithelial cells and mild to moderate tubular degeneration (Marshall, 1976; Sitpirija *et*

al., 1980)

(5) Observation of leptospires strand which spanned across the degenerated epithelial cell's cytoplasm and the tubular lumina (Marshall, 1976; Sitpirija *et al.,* 1980).

(6) Absence or presence of inflammatory cells such as lymphocytes, macrophages, and few neutrophils, surrounding the infected tubules (Marshall, 1976; Sitpirija *et al.,* 1980; Yang *et al.,* 2001)

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3.2.11.2 Observation /criteria for chronic infection

(1) Severe degeneration and necrosis of the tubular epithelial cells

(2) Leptospire colonies closely attached to the tubular epithelial cells within the lumen (Yener and Keles, 2001; Tuncuduva *et al.,* 2007)

(3) Presence of lymphoplasmacytic cellular infiltration (Tuncuduva *et al.,* 2007)

(4) Evidence of tubular epithelial cell regeneration such as cytomegaly, binucleation (Tuncuduva *et al.,* 2007).

(5) Absence of lymphoplasmacytic cellular infiltration (Tuncuduva *et al.,* 2007).

3.2.12 Statistical analysis

Percentages of both positive and negative cases were determined for different levels of age, year, sex and breed.These data were summarised using proportions with 95% confidence intervals calculated using the Wilson method as described by Brown *et al*. (2001).

Data were also represented using bar-charts. One way Analysis of variance was used to determine the significant difference in the haematological parameters. Student t-test was used to determine the significance between infected and non-infected and their observed microscopic lesions, performed by Analyse-it for Microsoft excel (Analytical software,). P-value <0.05 was considered statistically significant.

3.3 RESULTS

3.3.1 Incidence of suspected cases of leptospiral infection in dogs between 2003 to 2010 in two Veterinary Teaching Hospitals in the southwestern states of Nigeria

Clinical data of five hundred and seventeen suspected cases of canine leptospirosis, parasitic infections and normal dogs were used in this study. This was carried out between 2003 to 2010 in two Veterinary Teaching Hospitals in Southwestern Nigeria. The percentage yearly distribution of dogs within the period of study is depicted in Figure 3.1.

Within this period, a total of 517 cases of dogs were examined, out of which 188 cases (31.4%, 95% CI from 27 to 35%) were suspected of canine leptospirosis compared with 256 cases (42.8%, 95% CI from 39 – 47%) of other disease conditions such as babesiosis, erlichiosis e.t.c and 154 cases (25.8%, 95%CI from 22 to 30%) of normal dogs brought to these Veterinary Teaching hospitals for routine vaccination programmes. During this period, the incidence of dogs suspected of canine leptospirosis increased steadily from 16 dogs (2.7%, 95%CI from 02 – 04%) in year 2003 to 35 dogs (5.8%, 95%CI from 04 – 08%) in year 2007. The cases of other conditions brought to these two Hospitals at this period were more than the number of cases suspected of canine leptospirosis (from year 2003 to year 2010). The year 2003, recorded the lowest number of leptospiral suspected cases (16, 2.7%, 95%CI from 02 – 04%), while year 2007 had the highest number of suspected cases of leptospirosis (35, 5.8%, 95%CI from 04% to 08%). Moreover, from year 2007 to 2010 , there was no difference in the prevalence of suspected canine leptospirosis compared with the previous years.

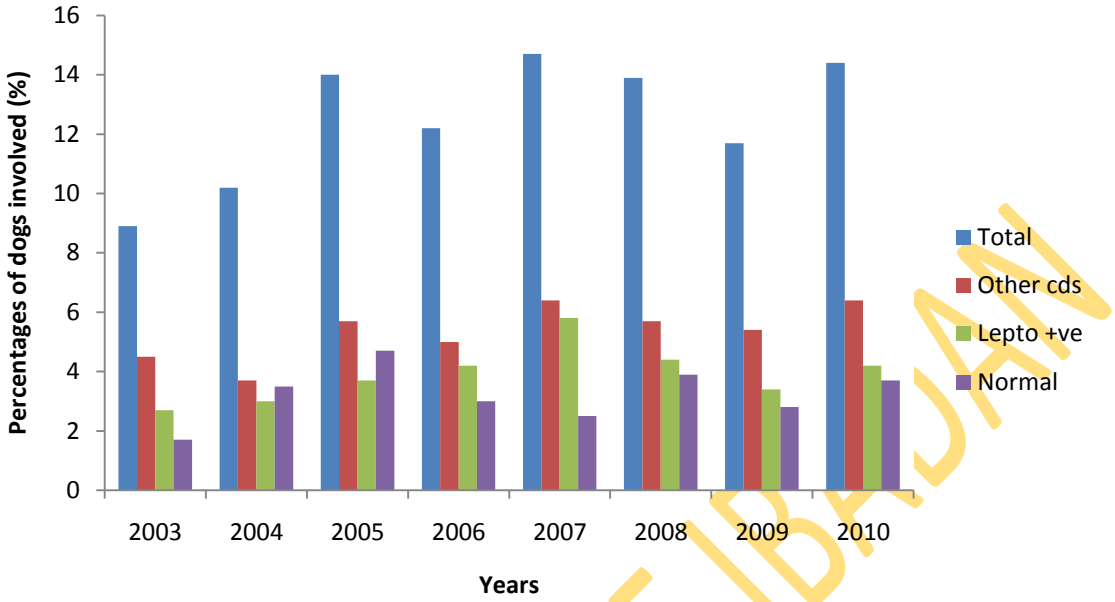

Fig.3.1. Bar-chart showing the percentages of dogs involved with suspected leptospirosis between 2003 to 2010 in the two Veterinary Teaching Hospitals.
Note: Other cds= other canine diseases, Lepto +ve =Leptospire positive

3.3.2 The prevalence of canine leptospirosis according to Age and Sex from two Veterinary Teaching Hospitals in southwestern Nigeria.

Table 3.1 depicts the age group of dogs suspected of canine leptospirosis. The age of these animals were divided into 3 categories (≤ 1 yr, 1-4 yrs and ≥4 years) and undetermined or unclassified age group. Dogs less than 1 year of age accounted for 147 (28.5%, 95%CI from 25 to 33%) out of 515 of all cases of dogs sampled. Out of the 147 dogs, 59 (33%, 95%CI from 26 to 40%), dogs were suspected of leptospirosis, while other conditions such as canine babesiosis and erlichiosis accounted for 88 cases (59.9%, 95%CI from 52 – 68%) within this period.

Dogs between 1-4 years were more susceptible to leptospiral infection than other age groups considering 72 dogs (40%, 95%CI from 34 to 48%) out of the 201 cases (39%, 95%CI from 35 to 43%) recorded for this age group, compared with 30 dogs (17%, 95%CI from 5 to 13%) suspected of canine leptospirosis of the 95 dogs (18.5%, 95%CI fromm 16 to 22) in the ≥4 years of age. The ages of 72 (14%, 95%CI from 11 to 17%) dogs out of the 515 dogs examined were undetermined from which 16 dogs (9%, 95%CI from 5 to 13%) were suspected of leptospiral infection.

The sex distribution of cases of suspected canine leptospirosis and other parasitic infections is depicted in Table 3.2.

There was a tendency toward an increased susceptibility rate in males dogs compared with the females. Out of the 515 dogs examined based on sex, 152 dogs (29.6%, 95%CI from 26 to 34) were females, while 178 (34.6%, 95%CI from 31 to 39) dogs were males, and the sexes of the remaining 184 dogs were undetermined. Out of the 152 female dogs, 64 (42.1%, 95%CI from 34 to 50) dogs were suspected of canine leptospirosis, compared with 178 male (34.6%, 95%CI from 31 to 39) dogs of which 79 males (44.4%, 95%CI from37 to 51) dogs were suspected of leptospiral infection. Out of the undetermined 184 cases (38.8%, 95%CI

from 32 to 40%), 34 cases (18.5%, 95%CI from 13 to 25) were suspected of canine leptospirosis.

3.3.3 Incidence of suspected cases of canine leptospirosis according to breed from 2003 to 2010 from two Veterinary Teaching Hospitals in south western Nigeria.

Thirteen different breeds of dogs were represented in the 504 cases examined in this study (Table 3.3). German Shepherd dog was the most common significant suspected breed with 278 cases (55.2%, 95%CI from 51-59%) out of which 107 cases (38.5%, 95%CI from 33 to 45%) were suspected of leptospiral infection. This was followed by Rottweiler with 66 cases (13.1%, 95%CI from 10 – 16%), out of which 36 (54.5%, 95%CI from 43 to 67%) cases were suspected of canine leptospirosis. Cross or mixed dogs were 11 (27.5%, 95%CI from 23 to 33%) out of 40 cases examined, local breed was 6 (25%, 95%CI from 20 to 30%) out of 24 cases, while bull mastiff was 5 (33.3%, 95%CI from 28 to 38%) out of 15 cases and Boer bull with 4 (30.8%, 95%CI from 26 to 36%) suspected cases out of 13 cases examined for leptospire infection. Three cases each (0.6%, 95%CI from 0 – 02%) of Boxer, Dobermann and Dalmatian were examined, out of which 2 (66.7%, 95%CI from 62 – 72%) were suspected of leptospiral infection in each cases. One Rigdeback, Great Dane and Lhasa apso (0.2%, 95%CI from 0.2 – 0.4%) were examined and suspected of canine leptospirosis.

Table 3. 1 Distribution of dogs with suspected and negative leptospirosis according to age groups from two Veterinary Teaching Hospitals in southwestern states of Nigeria (Between 2003- 2010)

		95%CI			95%CI			95%CI	
Years	Total	%	Lower - Upper	Leptospire negative	%	Lower - Upper	Leptospire suspect	%	Lower - Upper
0 – 1	147	28.5	25 – 33	88	59.9	52 - 68	59	33.3	26 - 40
1 – 4	201	39	35 – 43	129	64	57 – 71	72	40.7	34 – 48
< 4	95	18.5	16 – 22	65	68	59 – 77	30	17.0	11 – 23
UND	72	14	11 - 17	56	78	68 – 88	16	9	05 – 13

UND = undetermine age

Table 3. 2 Distribution of dogs with suspected and negative leptospirosis according to sex from two Veterinary Schools in southwestern states of Nigeria (Between 2003- 2010)

			95%CI			95%CI			95%CI
	Total	%	lower - upper	Leptospire negative	%	lower - upper	leptospire suspect	%	lower – upper
Female	152	29.6	26 – 34	88	57.9	50 – 66	64.0	36.2	34 – 50
Male	178	34.6	31 – 39	99.0	55.6	49 - 63	79.0	44.6	37 – 51
UNS	184	35.8	32 – 40	150.0	81.5	76 – 88	34.0	19.2	13 – 25

UNS= Undetermine sex .

3.3.4 Presenting clinical signs in suspected cases of canine leptospirosis between 2003 to 2010.

A wide range of clinical manifestions was reported on submission forms that accompanied cases suspected of leptospirosis to the laboratory (Table 3.4). From the clinical data available for 116 (23%, 95% CI of 19% to 27%) out of the 505 dogs examined in this study, numerous combination of signs and symptoms were documented. The most common clinical presentation involved non-specific overt signs such as pyrexia in 84 (72.4%, 95%CI from 64 to 80%) out of the 116 cases, while anorexia was recorded in 82 (70.7%, 95%CI, 63 to 78%) cases. This was followed by depression in 46 dogs (39.7%, 95%CI from 31% to 49%), palor in 36 dogs (31%, 95%CI from 23-39%), jaundice in 35 dogs (30.2%, 95%CI from 22 to 38%), haematochezia in 31 dogs (26.7%, 95%CI from 19 – 35%). A few of the suspected cases had features such as conjunctivitis and vomiting in 28 dogs (24.1%, 95% CI of 16% to 32%) each, dehydration in 16 dogs (13.8%, 95% CI of 8% to 20%), weight loss in 16 dogs (13.8%, 95% CI of 8% to 20%), weakness in 15 dogs (12.9%, 95% CI of 7% to 19%), while epistasis in 10 dogs (8.6%, 95% CI of 4% to 14%), pedal oedema in 7 dogs (6%, 95% CI of 2% to10%), dypsnoea in 3 dogs (2.6%, 95% CI of 0% to 6%), heamatoptysis in 2 dogs (1.7%, 95% CI of 0% to 5%) and haematuria in 2 dogs (1.7%, 95% CI of 0% to 5%) were observed in the least number of cases in this study.

There was no significant difference in the clinical signs of dogs with suspected leptospirosis compared with those that were negative and diagnosed of other conditions.

Table 3. 3 Distribution of negative and suspected cases of canine leptospirosis according to breed. Data on percentages and 95% confidence intervals (95% CI) were also presented.

No	Breeds	Total	%	95%CI Lower - Upper	-Ve	%	95%CI Upper - Lower	+Ve	%	95%CI Lower - Upper
1	Rottweiler	66	13.1	10 - 16	30	45.5	34 - 58	36	54.5	43 - 67
2	Alsatian	278	55.2	51 - 59	171	61.5	56 - 68	107	38.5	33 - 45
3	Boxer	3	0.60	00 - 02	1	33.3	02 - 64	2	66.7	62 - 72
4	Doberman	3	0.60	0.2 - 02	1	33.3	02 - 64	2	66.7	62 - 72
5	Dalmatian	3	0.60	0.2 - 02	1	33.3	02 - 64	2	66.7	62 - 72
6	Bull Mastiff	15	3.00	01 - 05	10	66.7	43 - 91	5	33.3	28 -38
7	Boer bull	13	2.58	01 - 05	9	69.2	44 - 94	4	30.8	26 – 36
8	Cross/mongrel	40	7.94	06 - 10	29	72.5	59 -87	11	27.5	23 – 33
9	Local	24	4.76	03 - 07	18	75.0	58 - 92	6	25.0	20 - 30
10	Pit bull	2	0.40	0.2 - 01	-	0.00	00 - 00	2	100	20.7 – 100
11	Ridgeback	1	0.20	0.2 - 0.4	-	0.00	00 - 00	1	100.0	20.7 - 100
12	GreatDane	1	0.20	0.2 – 0.4	-	0.00	00 - 00	1	100.0	20.7 - 100
13	Ihasa Apso	1	0.20	0.2 – 0.4	-	0.00	00 - 00	1	100.0	20.7 – 100
14	UNB	54	10.71	08 - 14	29	53.7	36 - 72	25	46.3	41 – 51

UNB = Undetermine breed, - = absent, -ve = leptospiral negative, +ve = leptospiral suspected

Table 3. 4 Distribution of dogs with negative and suspected leptospirosis showing specific clinical signs.

			95%CI			95%CI
Clinical signs	Number negative	%	Lower Upper	Number suspected	%	Lower - Upper
Anorexia	34	29.3	21 - 37	82	70.7	63 - 78
Vomiting	88	75.9	68 - 84	28	24.1	16 - 32
Dehydration	100	86.2	80 - 92	16	13.8	08 - 20
Weight loss	100	86.2	80 - 92	16	13.8	08 - 20
Dsypnoea	113	97.4	94 - 100	03	02.6	00 - 06
Haematochexia	85	73.3	65 - 81	31	26.7	19 - 35
Abdominal pain	110	94.8	91 - 99	06	05.2	01 - 09
Pyrexia	32	27.6	20 - 36	84	72.4	64 - 80
Depression	70	60.3	51 - 69	46	39.7	31 - 49
Jaundice	81	69.8	61 - 78	35	30.2	22 - 38
Epistasis	106	91.4	86 - 96	10	08.6	04 - 14
Conjuntivitis	88	75.9	68 - 84	28	24.1	16 - 32
Haematoptysis	114	98.3	95 - 100	02	01.7	00 - 05
Haematuria	114	98.3	95 - 100	02	01.7	00 - 05
Palor	80	69.0	61 - 77	36	31.0	23 - 39
Pedal oedema	109	94.0	81 - 93	07	06.0	02 - 10
Weakness	101	87.1	90 - 98	15	12.9	07 - 19

Table 3. 5 Distribution of pyrexia among the 136 suspected leptospiral positive and negative dogs.

	38.5 - 39.4^{0}C	%	95%CI Lower - Upper	39.5 - 40.4^{0}C	%	95%CI Lower – Upper	40.5 - 41.4^{0}C	%	95%CI Lower - Upper
Lepto +ve	23/84	27.4	17.0 – 37.0	38/84	45.2	34.0 – 56.0	23/84	27.4	17.0 – 37.0
Lepto-ve	13/52	25.0	13.0 – 37.0	32/52	61.5	49.0 – 75.0	7/52	13.5	05.0 – 23.0

3.3.5 Haematological profiles of suspected cases of canine leptospirosis between 2003 and 2010

The haematological profiles of dogs suspected of canine leptospirosis, others diseases (with conditions such as babesiosis, helminthosis, erhlichiosis) and normal dogs are represented in Table 3.6. Out of the archives of the two Veterinary Teaching Hospitals, haematological profiles of 279 dogs were obtained. Out of these, 122 cases were suspected of canine leptospirosis, 99 cases were leptospiral negative (with conditions such as babesiosis, helminthosis, erhlichiosis) and 58 cases of dogs brought to these Teaching Hospitals for routine vaccination were regarded as normal, were selected for this study.

Complete blood count performed from the blood drawn on presentation showed that anaemia was apparent in both leptospiral suspected and leptospiral negative dogs, evidenced by low level of the mean values of erythrocyte indices in both cases; (leptospiral suspected dogs PCV= 32.17±12.84% ranged from 09 to 60.0%, Hb= 10.07±4.28g/dl ranged from 2.40 to 19.70g/dl, RBC=05.12±2.08 $x10^3/mm^3$ ranged from 1.02 to9.65 $x10^3/mm^3$ and leptospiral negative dogs with PCV =32.00±10.77%, ranged from 9.0 to 55.0%, Hb= 9.91±3.63g/dl, ranged from 1.20 to 18.4g/dl and RBC= 5.48±2.08 $x10^3/mm^3$ ranged from 0.91 to 13.70 $x10^3/mm^3$) compared with the normal (PCV= 43.17±7.82%, Hb=13.62±2.73g/dl and RBC= 7.12±1.39 $x10^3/mm^3$). The anaemia in the leptospiral suspected and leptospiral negative cases was characterized by normocytic hypochromic and slight microcytic normochromic anemia respectively (MCV=64.02±02fl, MCHC=30.67±3.65g/dl and MCV=58.72±9.70fl, MCHC=30.87±3.71g/dl respectively) compared with the normal dogs (MCV=61.53±10.28fl, MCHC=31.44±2.25g/dl). The mean value of the MCV in the leptospiral negative dogs showed significant decrease ($p<0.05$) compared with the leptospiral suspected and normal dogs.

The mean value of the WBC count in the leptospiral suspected dogs was at the upper limit of the normal compared with the normal leukogram. There was moderate significant neutropilia with left shift in both leptospiral suspected (neutrophil=10.80±65.98 $x10^3/mm^3$ and band=0.93±12.43 $x10^3/mm^3$) and negative (neutrophil=7.18±42.37 $x10^3/mm^3$ and band= 0.41 ± 0.36 $x10^3/mm^3$) cases, although it was more in the leptospiral suspected cases compared with the normal cases (neutrophil= 6.99±24.16 $x10^3/mm^3$ and band=0.28±0.22 $x10^3/mm^3$). There was no significant change in the mean values of the lymphocytes in the leptospiral suspected and negative cases compared with the normal dogs. Significant monocytosis in the leptospiral suspected cases (monocyte=0.59±0.51 $x10^3$/mm3) was observed compared with the leptospiral negative and the normal dogs (monocyte=0.38±0.30 $x10^3/mm^3$ and 0.32 ±0.25 $x10^3/mm^3$ respectively). Mild significant eosinophilia was also observed in the leptospiral suspected cases (eosinophil = 0.59±0.51 $x10^3/mm^3$) but was more in the leptospiral negative cases (eosinophil =0.70 ±0.79 $x10^3/mm^3$) compared with the normal dogs (eosinophil =0.44 ±0.27 $x10^3/mm^3$). There was also moderate significant thrombocytopenia (platelet =176.2S3±85.04 $x10^3/mm^3$) in the leptospiral suspected cases compared with the leptospiral negative and normal dogs (platelet = 230.91±86.72 $x10^3/mm^3$ and 219.90±62.50 $x10^3/mm^3$ respectively).

Table 3. 6 Comparative haematological profiles of normal, negative and suspected cases of canine leptospirosis from the two Veterinary Teaching Hospitals between 2003 to 2010.

Parameters (number examined)	Total (n=279)	95%CI Lower-Upper	lepto +ve (n=122)	95%CI Lower-Upper	Lepto –ve (n=99)	95%CI Lower-Upper	Normal haemogram (n= 58)	95%CI Lower-Upper	Reference range
Hb (g/dl)	10.75±4.04*	10.27 – 11.23	10.07±4.28[b]	9.3 – 10.83	9.91±3.63[b]	09.18 – 10.63	13.62±2.73[a]	12.90 – 14.34	13.3-19.2
PCV (%)	34.45±12.06[b]	33.04 – 35.86	32.17±12.84[b]	2.88 – 34.46	32.00±10.77[b]	01.08 – 29.85	43.17±7.82[a]	41.15 – 45.19	36.0-54.0
RBC($x10^3/mm^3$)	5.68±2.10[a]	5.43 – 5.93	05.12±2.08[b]	4.74 – 5.49	5.48±2.08[b]	05.05 – 05.91	7.12±1.39[a]	06.76 – 07.48	05.6-8.0
MCV(fl)	61.69±10.81[a]	60.40 – 62.97	64.02±02[a]	61.97 – 66.07	58.72±9.70 [b]	56.72 – 60.73	61.53±10.28[a]	58.87 – 64.18	60.0-75.0
MCHC(g/dl)	30.90±3.43	30.50 – 31.31	30.67±3.65	30.01 – 31.32	30.87±3.71	30.12 – 31.61	31.44±2.25	30.86 – 32.03	34.0-38.0
WBC($x10^3/mm^3$)	12.90±75.05[a]	11.99 – 13.76	15.17±77.64[c]	13.78 – 16.55	10.87±50.30[b]	09.86 – 11.88	11.45±90.46[a]	9.10 – 13.81	06.4-15.9
Segm. ($x10^3/mm^3$)	8.72±5.46	08.08 – 09.36	10.80±65.98[c]	09.62 – 11.98	7.18±42.37[b]	06.33 – 08.02	6.99±24.16[a]	06.37 – 07.62	43.0-88.0
Band ($x10^3/mm^3$)	0.64 ±0.93[b]	0.52 – 0.76	0.93±12.43[c]	0.70 – 01.17	0.41 ± 0.36[b]	0.33 – 0.50	0.28±0.22[a]	0.22 – 0.36	0.0-0.45
LYM ($x10^3/mm^3$)	2.60 ±1.78	2.39 – 2.80	02.76±1.82	02.44 – 03.90	2.44 ±1.63	02.12 – 02.76	2.51±1.89	02.02 – 03.00	02.8-36.0
Mon ($x10^3/mm^3$)	0.46±0.40[a]	0.41 – 0.51	0.59±0.51[b]	0.50- 0.68	0.38±0.30[a]	0.32 – 0.45	0.32 ±0.25[a]	0.24 – 0.39	02 - 11.0
EOS ($x10^3/mm^3$)	0.60±0.61	0.51 – 0.68	0.59±0.51[b]	0.47 – 0.70	0.70 ±0.79[c]	0.53 – 0.88	0.44 ±0.27[a]	0.36 – 0.52	0-17
PLAT ($x10^3/mm^3$)	242.88±119.2[a]	227.7- 258.04	176.23±85.04[c]	159.26 -193.20	230.91±86.72[a]	212.00 – 250.00	219.90±62.50[a]	202.50 – 237.30	186-547

**Numbers with the same superscript are not significantly different, lepto +ve = leptospiral suspected, lepto –ve = leptospiral negative*

3.3.6 Biochemical changes in dogs with leptospirosis

The Table 3.7 below depicts the biochemical changes observed in dogs leptospirosis. Blood urea nitrogen (BUN) levels were elevated in 3 (42.9%) dogs out of the 7 dogs ranging from 31.4 -60.9mg/dl. However, creatinine levels were elevated only in 2 dogs (28.6%) and the remaining dogs were within the normal reference values (1.0 - 1.1 mg/dl compared with 0.5 - 1.7 mg/dl normal reference value) indicating both renal and pre-renal azotemia. Four (57.14%) dogs had hyperbilirubinemia out of the 7 dogs ranging from 1.1 to 19.9mg/dl (normal range = 0.0 to 0.3mg/dl). Alkaline phosphatase levels in 3 dogs (42.9%) were elevated ranging from 116.7 to 412mmol/L compared with the normal reference value (1 - 114mmol/L). There was also increase in the serum ALT levels in 2 (28.6%) dogs (110.0µl/L and 176µl/L) and the remaining 5 dog's values were at the upper limit of the normal reference values. Only 1 (14.3%) dog showed hypernatremia (155.0 mmol/L) while other dogs were within the normal reference values. Hypokalemia was observed in 1 (14.3%) dog, while other dogs were within the reference range. Hypochloremia in 2 (28.6%) dogs and hyperchloremia in 1 (14.3%) dog.

Table 3. 7 Biochemical profiles of dogs with canine leptospirosis from the two Veterinary Teaching Hospitals between 2003 to 2010.

Parameters	(Naff) Mean±S.E	Min. - Max	Reference values*
Urea (mg/dl)	(3) 34.0±7.96	14 – 60.9	8-28
Creatinine (mg/dl)	(2) 1.5±0.37	1.0 – 3.0	0.5-1.7
Total Protein(g/dl)	6.03±0.62	3.7 – 8.5	6.0-7.5
Albumin (g/dl)	1.30±0.48	0.5 – 4.60	2.3-3.1
Globulin (g/dl)	3.52±0.81	1.8 – 8.0	2.7-4.4
Total bilirubin (mg/dl)	(4) 6.34±3.60	1.1 – 19.9	0.0-0.3
ALT(µ/L)	(2) 93.95±19.9	29.0 – 176	10-109
AST(µ/L)	54.92±29.28	13 - 200	13-15
ALP (µ/L)	(3)146.98±54.07	60.2 - 412	1-114
Sodium(mEq/L)	145.50±2.60	142 - 153	142-152
Potassium(mEq/L)	3.95±0.32	3.0 – 44	3.9-5.1
Chloride (mEq/L)	88.60±23.87	109 - 170	110-124
Bicarbonate (mEq/L)	21.00±1.29	18 - 24	17-24

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3.3.7 Gross pathology of suspected cases of canine leptospirosis between 2003 – 2010.

Pathomorphological observations and the number of dogs involved with the degree of the morphological alteration are depicted in Tables 3.8 and 3.9. Out of the 104 dogs examined in this study, 34 (46%, 95%CI from 35 – 57%) and 18 (24.3%, 95%CI from 14 – 34%) cases showed dehydration and conjunctivitis respectively. There was moderate to severe subcutaneous jaundice in 36 (48.7%, 95%CI from 38 – 60%) dogs (Figs.3.2-3). Mild to moderate pulmonary congestion and oedema was observed in 48 (64.9%, 95%CI from 54 – 76%) cases and 39 (52.7, 95%CI from 42 – 64%) dogs showed pulmonary haemorrhages (both petechial and ecchymotic) in the lungs (Fig.3.12). Mild to severe haemorrhagic gastroenteritis was observed in 45 (60.8%, 95%CI from 50 – 70%) dogs with multiple foci of both petechial and ecchymotic haemorrhages and frank blood along the small and the large intestine (Figs.3.4-5). Slight splenomegaly was observed in 1 case (1.4%, 95%CI from 0 – 3%) and moderate to severe splenic atrophy in 16 (21.6%, 95%CI from 13 -31%) dogs. There was severe lymphadenopathy of the various lymphoid tissues in all the 74 dogs with 31 (41.9%, 95%CI from 31 – 53%) dogs showing haemorrhagic and siderotic lympadenopathy, especially in the mesenteric, mandibular, mediastinal, prescapular and the femoral lymph nodes. There was also mild to moderate hepatomegaly with jaundice in 46 (62.5%, 95%CI from 51 – 73%) dogs. Renal haemorrhages in 23 (31.1%, 95%CI from 20 – 42%) and multiple foci of cortical necrosis with some showing (31, 41.9%, 95%CI from 31 – 53%) pitted, rough and granular cortical surfaces. Eight (10.8%, 95%CI from 4 – 18%) dogs showed multiple foci of petechial haemorrhages in the pancreas. Moderate to severe congestion of the meningeal and cerebral blood vessels were also observed in 26 dogs (35.1%, 95%CI from 24 -46%) (Fig.3.6). Gross pathological changes such as mild to moderate pedal oedema in 5 (6.8%, 95%CI from 1-13%) dogs, mild heamorrhagic cystitis in 1 (1.4%, 95%CI from 0-3%) and mild to moderate myocardial haemorrhage in 17 (23%, 95%CI from 13-33%) were also observed in this study.

Table 3. 8 Gross morphological changes in104 dogs with suspected of canine leptospirosis

Morphological changes	Lepto-ve n=30	%	Lepto+ (n=74)	%	95% CI Lower	Upper
Dehydration and emaciation	23	76.7	34	46.0	35	57
Conjunctivitis	21	70	18	24.3	14	34
Subcutaneous jaundice	14	46.7	36	48.7	38	60
Pulmonary congestion and oedema	21	70	48	64.9	54	76
Pulmonary haemorrhage	17	56.7	39	52.7	42	64
Heamorrhagic gastroenteritis	16	53.3	45	60.8	50	72
Splenomegaly	25	83.3	01	01.4	00	03
Splenic atrophy	5	16.7	16	21.6	13	31
Haemorrhagic lymphadenopathy	12	40	31	41.9	31	53
Hepatomegaly with jaundice	21	70	46	62.2	51	73
Renal haemorrhages	2	6.7	23	31.1	20	42
Renal cortical necrosis	8	26.7	31	41.9	31	53
Meningeal congestion and oedema	12	40	26	35.1	24	46
Pedal oedema	1	3.3	05	06.8	01	13
Pancreatic heamorrhage	1	3.3	08	10.8	04	18
Haemorrhagic Cystitis	3	10	01	01.4	00	3
Myocardial haemorrhage	6	20	17	23.0	13	33

Table 3. 9 The degree of gross morphological changes and the number of cases involved in the 74 dogs suspected of leptospira infection in the two Veterinary Teaching Hospitals

	No of cases	Degree of the lesions			
Lesions		-	+	++	+++
Dehydration and emaciation	34/74	ND	ND	ND	ND
Conjunctivitis	18/74	ND	ND	ND	ND
Subcutaneous jaundice	36/74	-	17	14	05
Pulmonary congestion and oedema	48/74	-	08	22	18
Pulmonary haemorrhage	39/74	-	05	24	10
Heamorrhagic gastroenteritis	45/74	-	17	19	09
Splenomegaly	01/74	-	01	-	-
Haemorrhagic lymphadenopathy	31/74	-	ND	ND	ND
Hepatomegaly with jaundice	46/74	-	11	32	03
Renal haemorrhages	23/74	-	12	06	05
Renal cortical necrosis	31/74	-	17	12	02
Meningeal congestion and oedema	26/74	-	15	11	-
Pedal oedema	05/74	-	01	04	-
Pancreatic heamorrhage	08/74	-	05	03	-
Haemorrhagic Cystitis	01/74	-	01	-	-
Myocardial haemorrhage	17/74	-	09	08	-

+ =mild, ++ = moderate, +++ = marked, ND = Not determine.

Fig. 3.2. Photograph of one carcass showing severe subcutaneous jaundice and ecchymotic and suffusion haemorrhages.

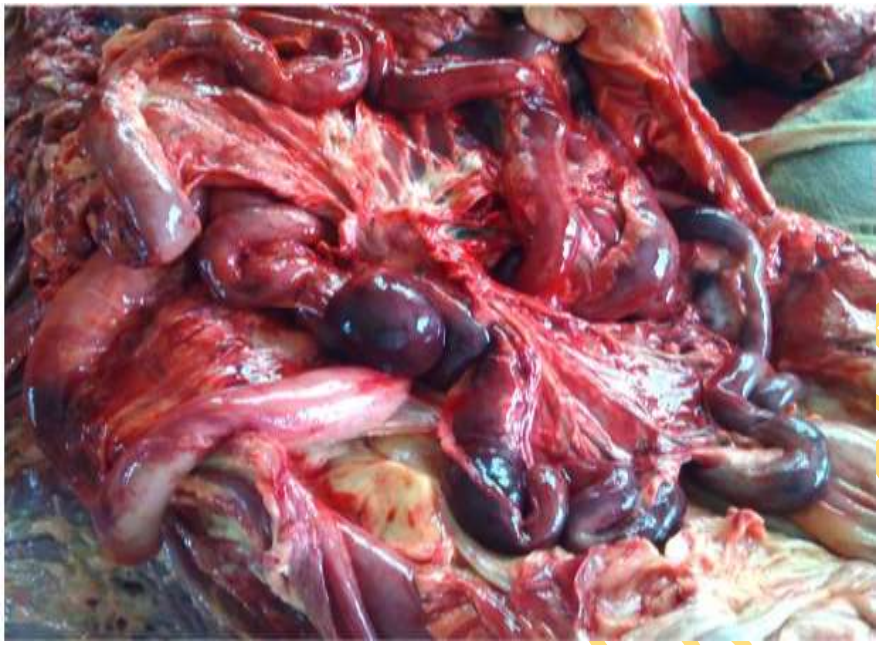

Fig.3.3. Photograph of the intestine of dog with leptospiral infection showing severe diffuse acute haemorrhagic enteritis.

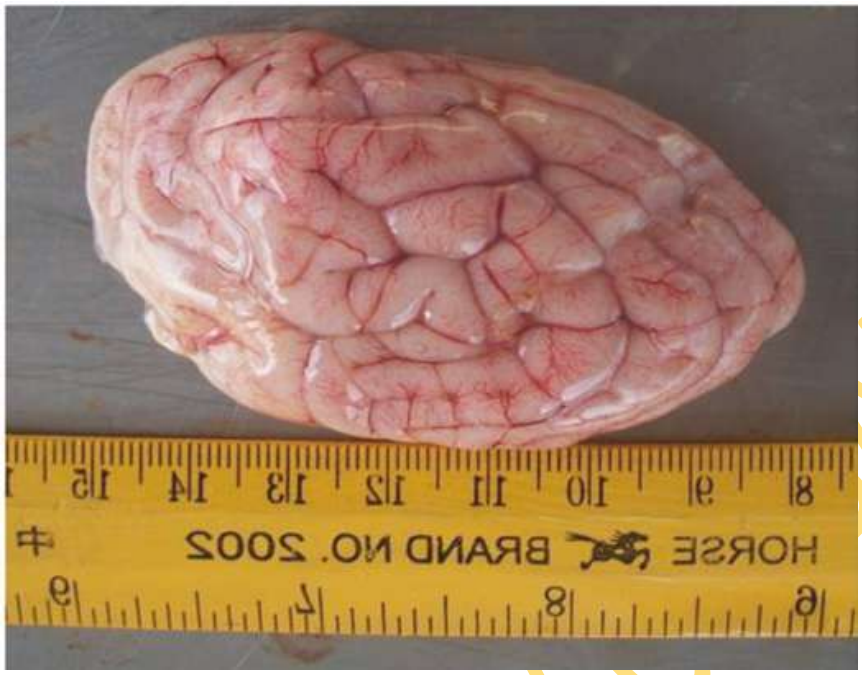

Fig.3. 4 Photograph of the brain of dog with leptospirosis showing markedly congested cerebral blood vessels.

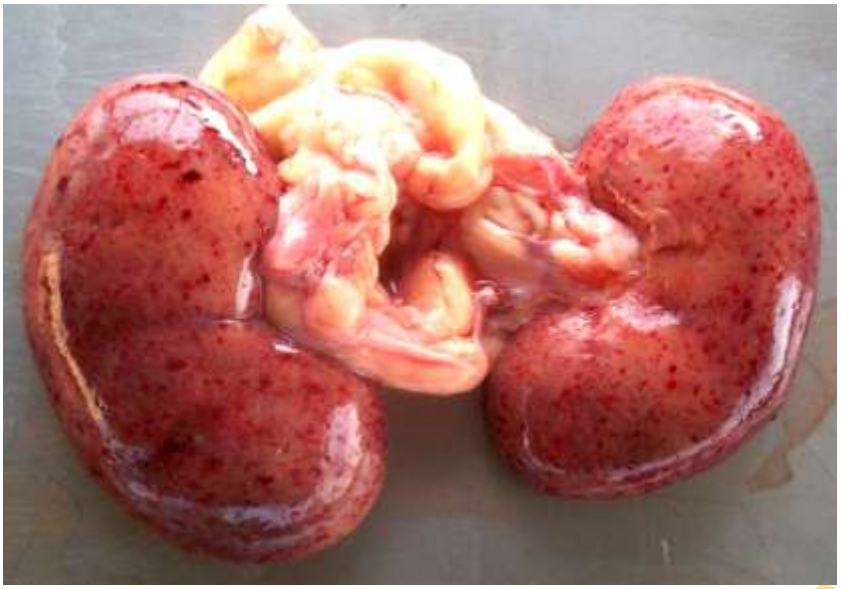

Fig.3. 5. Photograph of kidney of dog with leptospirosis showing marked widespread multiple foci of petechial and ecchymotic haemorrhages on the subcapsular with severe jaundice of the peri-renal fat.

3.3.8 Histopathology of the organs in leptospiral suspected dogs.

Apart from the kidney, organs such as liver, lung, heart, brain, pancreas and spleen and lymph nodes of 55 cases of dogs suspected of leptospirosis were evaluated for histopathological changes.

3.3.8.1 Liver

The microscopic features of the livers in the examined dogs revealed mild to marked focal or diffuse areas of distortion of hepatic cords in 49 (89.1%, 95%CI from 81 – 97%) cases with mild to moderate vacuolar degeneration in 48 cases (87.2%, 95%CI from78 – 96%). Twenty (36.4%, 95%CI from 23- 49%) out of the 55 cases showed mild to moderate focal areas of hepatocyte necrosis with mild to severe sinusoidal dilatation in 46 cases (83.6%, 95%CI from 74 – 96%) (Fig.3.8). Forty-eight cases (87.2%, 95%CI from 78-96%) showed mild to moderate diffuse activated kupffer cells, proliferation with haemosiderin and mild to severe erythrophagocytosis in 43 (78.2%, 95%CI from 67 – 89%) cases. Evidence of regeneration such as binucleation and cytomegaly were slightly or moderately present in all the cases examined (100%, 95%CI from 0 – 100%). There was also mild to moderate periportal oedema with mononuclear cellular infiltration at the portal tract in all the cases (100%, 95%CI from 0 – 100%) examined. With Warthin Starry silver stain, 3 liver sections showed the presence of leptospiral organism in the sinusoids (Fig.3.9). Immunohistochemical staining with the 6 rabbit monoclonal antibodies of the three liver sections revealed *leptospire interrogan* serovar *icterohaemorrhagiae* in only one of the liver (Fig 3.10a & b). The remaining 2 livers that demonstrated the presence of leptospiral organism with the WSSS did not show any reactivity to any of the six monoclonal antibodies. .

Table 3. 10 Number and severity of histopathological changes in different organs in 55 dogs suspected of canine leptospirosis from the two Veterinary Teaching Hospital.

	Severity and the number of cases involved					95%CI
Organs/Lesions	-	+	++	+++	Total (%)	Lower - Upper
Lung						
Pulmonary oedema and congestion	-	5/55	20/55	27/55	52 (94.6)	89.0 – 100.0
Pulmonary haemorrhage	3/55	7/55	21/55	8/55	36 (65.5)	53.0 – 79.0
Erythropagocytosis	2/55	11/55	22/55	3/55	34 (61.8)	49.0 – 75.0
Haemosiderosis	7/55	10/55	14/55	4/55	28 (50.9)	38.0 – 64.0
Thickened alveolar wall	-	9/55	28/55	8/55	45 (81.8)	72.0 – 92.0
Liver						
Loss of hepatic cohesion	6/55	25/55	20/55	4/55	49 (89.1)	81.0 – 97.0
Hepatic vacuolations	7/55	17/55	24/55	7/55	48 (87.2)	78.0 – 96.0
Hepatic necrosis	35/55	14/55	4/55	2/55	20 (36.4)	23.0 – 49.0
Sinusoidal dilatation	9/55	20/55	12/55	14/55	46 (83.6)	74.0 – 94.0
Kupffer cell proliferation	7/55	16/55	27/55	5/55	48 (87.2)	78.0 – 96.0
Erythrophagocytosis	12/55	23/55	20/55	-	43 (78.2)	67.0 – 89.0
Binucleation and cytomegaly	-	36/55	19/55	-	55 (100)	00.0 – 100.0
Periportal oedema	-	21/55	24/55	10/55	55 (100)	00.0 – 100.0
Periportal mononuclear cellular infiltration	-	17/55	23/55	15/55	55 (100)	00.0 – 100.0
Spleen and Lymph node						
Lymphoid hypoplasia	-	15/55	26/55	14/55	55 (100)	00.0 – 100.0
Erythroid hypoplasia	-	2/55	14/55	39/55	55 (100)	00.0 – 100.0
Prominent trabeculae	-	3/55	26/55	26/55	55 (100)	00.0 – 100.0
Thickened capsule	-	15/55	26/55	14/55	55 (100)	00.0 – 100.0
Erythrophagocytosis	-	12/55	23/55	20/55	55 (100)	00.0 – 100.0
Haemosiderosis	-	12/55	23/55	20/55	55 (100)	00.0 – 100.0
Haemorrhagic lymphadenitis	9/55	11/55	27/55	8/55	46 (83.6)	74.0 – 94.0
Pancreas						
Interstitial oedema and congestion	-	19/55	30/55	6/55	55 (100)	00.0 – 100.0
Interstitial haemorrhage	8/55	27/55	14/55	6/55	47 (85.5)	77.0 – 95.0
Pancreatic necrosis	12/55	19/55	11/55	13/55	43 (78.2)	67.0 – 89.0
Heart						
Interstitial oedema	21/55	19/55	12/55	3/55	34 (61.8)	49.0 – 75.0
Interstitial cellular infiltration	36/55	13/55	5/55	1/55	19 (34.6)	22.0 – 48.0
Brain						
Meningeal congestion and oedema	-	19/55	23/55	3/55	55 (100)	00.0 – 100.0
Neuronal degeneration	-	22/55	20/55	13/55	55 (100)	00.0 – 100.0

- = absent + = mild, ++ = moderate, +++ = severe

Table 3.11 Cultural isolation of leptospires in 44 kidneys and blood of dogs collected during postmortem suspected of leptospirosis according to sex

No		Total	%	95%CI Lower-Upper	Lepto+ve	%	95%CI Lower-Upper	Lepto-ve	%	95%CI Lower-Upper
1	Male	32	72.7	60 - 86	29	65.9	52 - 80	3	6.8	0 - 15
2	female	12	27.3	14 - 40	8	18.2	07 - 29	4	9	0 - 18

The staining appears as granular debris in the cytoplasm of hepatocytes, activated kupffer cells (Fig.3. 18) and the endothelium of the hepatic blood vessels.

3.3.8.2 Spleen and Lymph node

The spleen of the affected dogs showed mild to severe thickened (due to fibrous connective) and corrugated capsule, (12/12, 100%), with depletion of both red and white corpsules with prominent trabeculae, activation of reticuloendothelial cells, evidenced by mild to moderate diffuse erythrophagocytosis and haemosiderosis in all the 55 cases examined (100%, 95%CI from 0 – 100%). Erythrophagocytosis and haemosiderosis were more prominent in the sinuses of the lymph nodes in 46 cases (83.6%, 95%CI from 74 – 94%).

3.3.8.3 Lung

Fifty-two lungs (94.6%, 95%CI from 89-100%) out of the 55 affected dogs showed moderate to severe inflammatory exudate in the alveolar spaces and within the bronchioles. The pulmonary blood vessels were severely congested in 36 cases (65%, 95%CI from 53 – 79%) with mild to moderate pulmonary haemorrhages in the alveolar spaces (Fig 3.7). The alveolar wall was thickened in 45 cases (81.8%, 95%CI from 72 – 92%), evidenced by moderate increase in interstitial cellularity of the alveolar septae due to alveolar macrophages, lymphocytes and neutrophils. Moderate erythrophagocytosis by alveolar macrophages was observed in 34 cases (61.8%, 95%CI from 49 -75%) and mild to moderate heamosiderin-laden macrophages in 28 cases (50.9%, 95%CI 38 – 64%). Multiple foci of haemorrhages were observed in some cases while some coalesced to formed diffuse areas of haemorrhages.

3.3.8.4 Pancreas

There was mild to moderate vascular congestion and interstitial oedema in all the 55 cases (100%, 95%CI from 0 – 100%) examined, with mild to moderate multifocal or locally diffuse areas of haemorrhages in 47 cases (85.5%, 95%CI from 77-95%). Mild pancreatic

degeneration and necrosis were observed in 43 cases (78.2%, 95%CI from 67 – 89%) with diffuse but sparse mononuclear cellular infiltration in the interstitium (Fig.3.6).

3.3.8.5 Heart

Histopathological changes in the heart of affected dogs showed mild to moderate vascular congestion and interstitial oedema in 43 cases (61.8%, 95%CI from 49 – 75%) with slight heamorrhages. Mild to moderate interstitial mononuclear and polymorphonuclear cellular infiltration (mostly lymphocyte and neutrophils) in 19 cases (34.6%, 95%CI from 22 – 48%) examined.

3.3.8.6 Brain

The meningeal blood vessels of all the dogs examined showed mild to moderate congestion (100%, 95%CI from 0 – 100%). There was mild neuronal degeneration and gliosis. Non-suppurative inflammatory foci were also observed in few instances in the meninges and cerebral parenchymal.

3.3.9 Cultural isolation of leptospiral organism in the kidney of 41 dogs

Table 3.11 above depicts the cultural isolation of leptospiral organism from 41 kidneys of dogs. Out of the 41 kidneys, 29 (70.7%, 95%CI from 64 – 78%) and 12 (29.3%, 95%CI from 15 – 43%) were males and females respectively. Twenty six kidney samples (68.4%, 95%CI from 48 – 78%) were culturally positive to leptospiral infection out of the 29 male dogs in this study, compared with 3 (7.3%, 95%CI from 0-15%) negative male dogs, while 8 (19.5%, 95%CI from 8 – 32%) were positive out of the 12 female dogs examined, compared with 4 (9.8%, 95%CI from 1-19%) negative female dogs. In all, 34 (82.9%) kidneys sample were positive out of the 41 samples examined while 7 (17.1%) samples were negative.

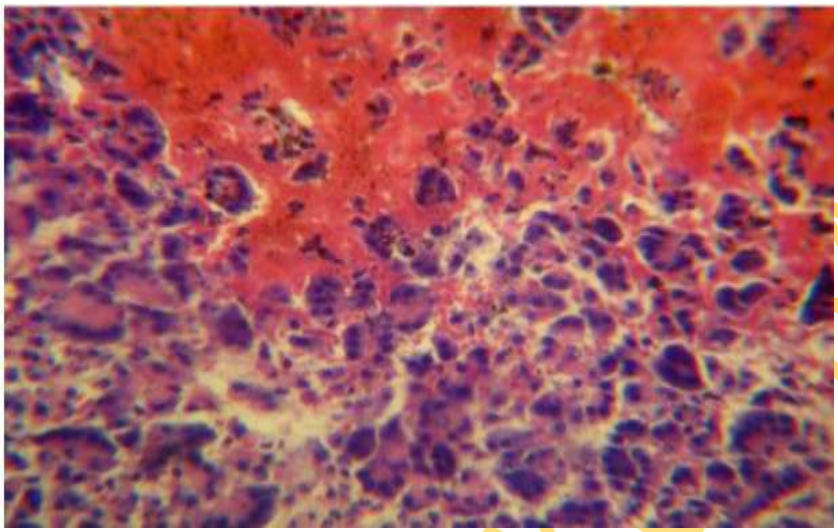

Fig.3.6 Photomicrograph of the pancreas showing locally extensive area of haemorrhages (H) with necrosis (N) and disorganisation of the pancreatic acinar. H & E. x 300,

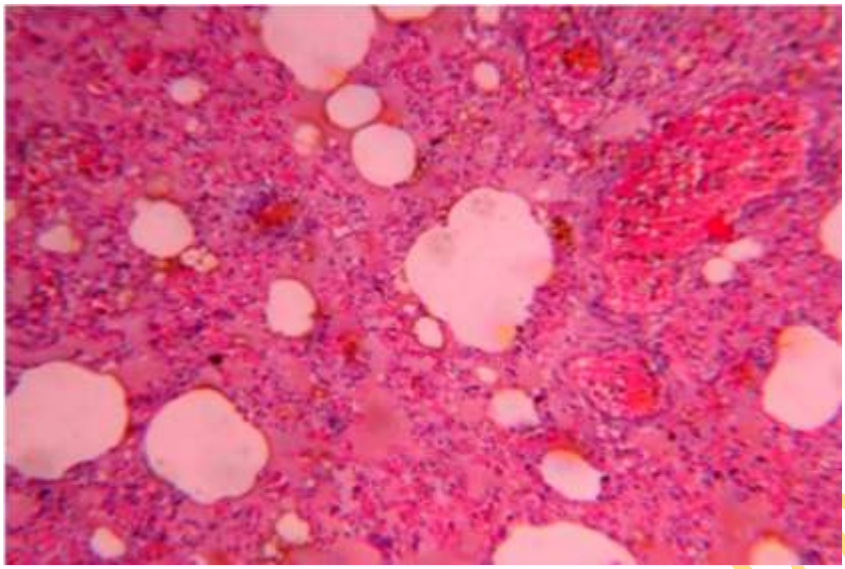

Fig.3.7 Photomicrograph of the lung section showing severe pulmonary congestion and inflammatory and cellular exudate both within the alveolar spaces. H & E. x 250

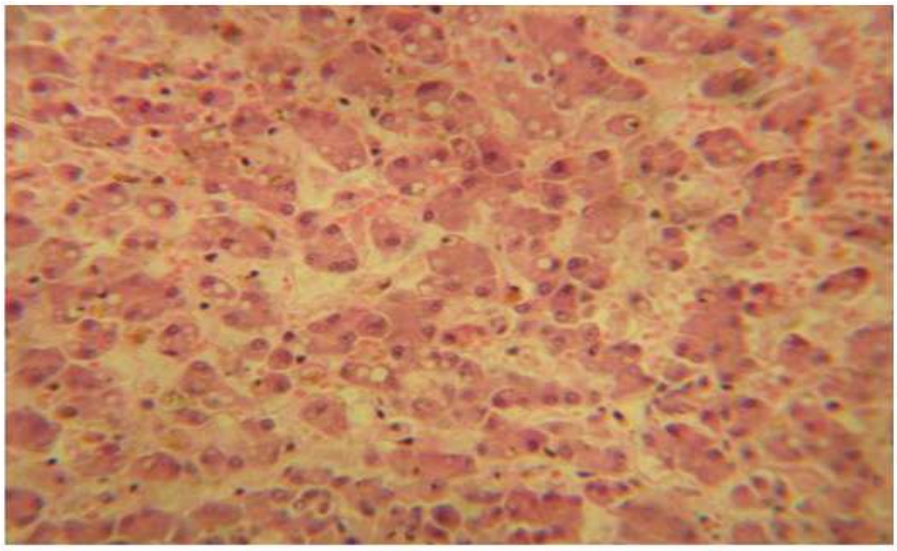

Fig.3.8 Photomicrograph of the liver of dog infected with *leptospire interrogans* serovar *icterohaemorrhagiae* showing severe dissociation and individualization of hepatic cords, sinusoidal dilatation and mild vacuolar degeneration. H & E. x 300

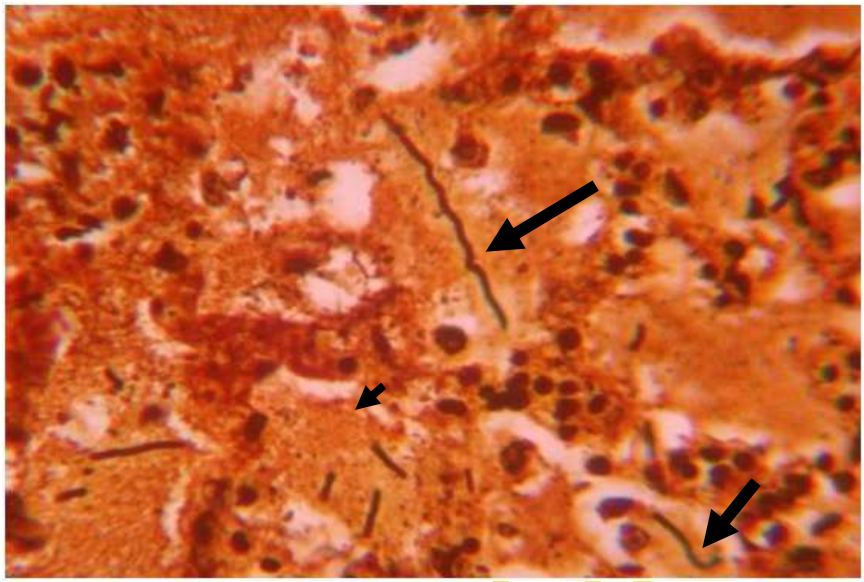

Fig.3. 9 Photomicrographs of liver sections showing the presence of leptospires (arrows) within the dilated sinusoids and adjacent hepatic cords. Warthin Starry silver stain. x 300.

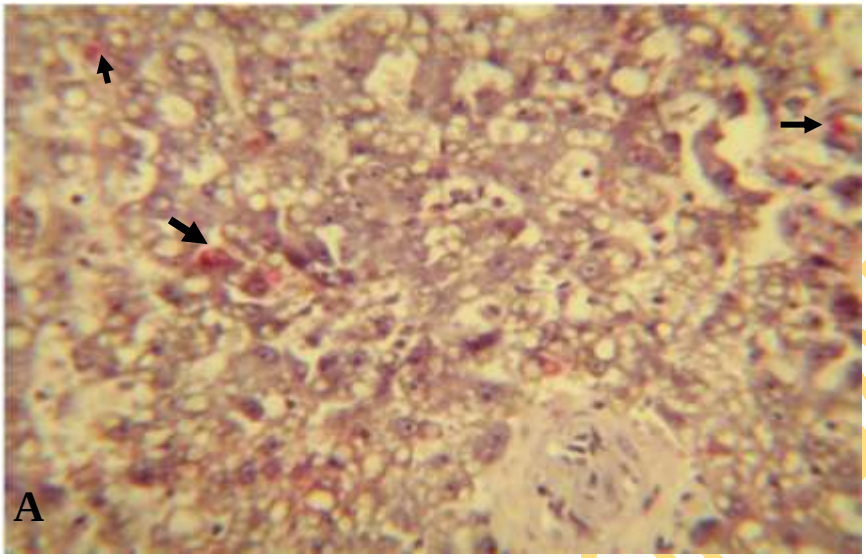

Fig.3.10 Immunohistochemical detection of *leptospira interrogans* serovar *icterohaemorrhagiae* in dog. Leptospiral debris and fragment are reddish stained in the cytoplasm of hepatocytes and sinusoids and kupffer cells (arrows)., Alkaline phosphatase-fast red, counterstained with heamatoxylin. x 300

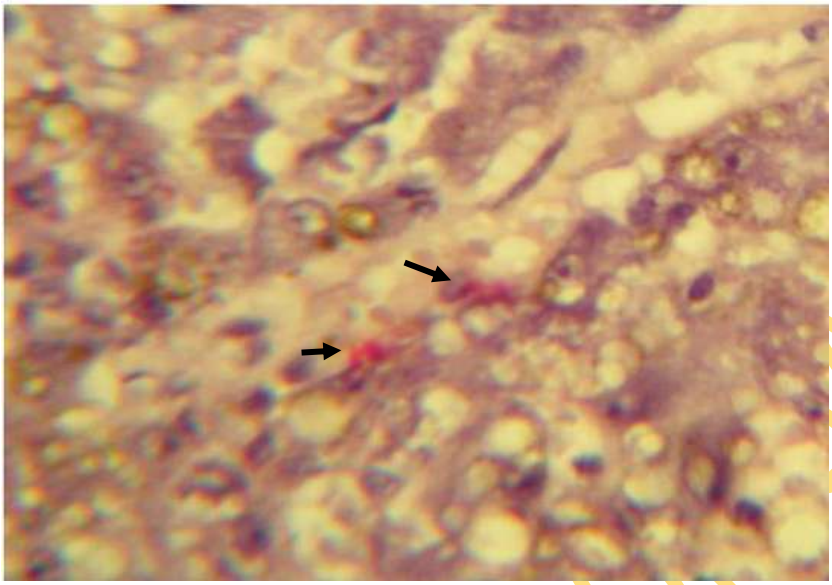

Fig.3.11 Immunohistochemical detection of *Leptospira interrogans* serovar *icterohaemorrhagiae* in dog. Leptospiral debris and fragment are reddish stained in the cytoplasm of endothelial cells (arrows). Alkaline phosphatase-fast red, counterstained with heamatoxylin. x 400

3.3.10 Detection of pathogenic leptospiral organism from organs and blood of suspected dogs by PCR.

Sixteen leptospiral isolates from dogs were subjected to PCR analysis. Out of the 16 samples, 2 were isolated from blood of dogs while the remaining 14 were from the kidneys of dogs submitted for postmortem examinations. DNA extracted from all the kidney and blood samples were subjected to PCR amplification, using G1/G2 specific primers for pathogenic leptospire. Out of the 16 samples only 5 (31.3%) showed amplicons of 285bp specific for pathogenic leptospiral interrogans genes. Eleven (68.8%) out of the 16 samples were negative (Figs.3.11 and 12). Out of the 5 positive samples, 1 of the isolate was from the blood of dogs with clinical sings of fever, anorexia and jaundice and the remaining 4 were isolated from the kidney of dogs brought for postmortem examination.

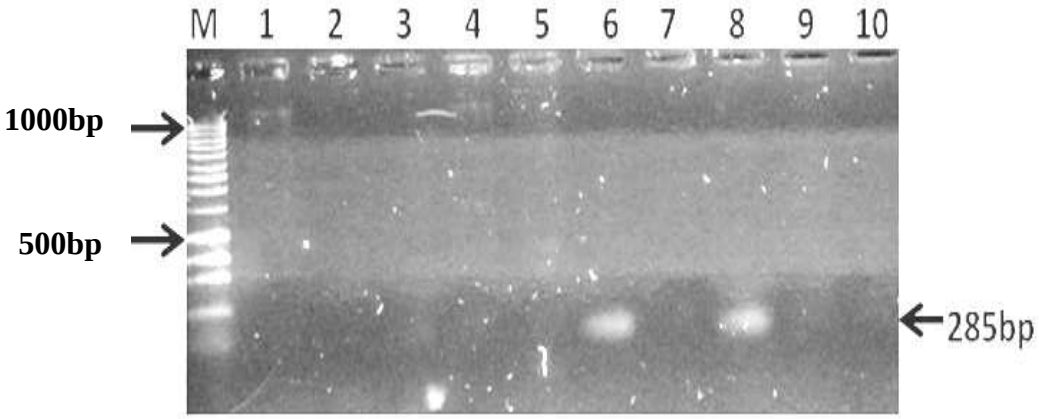

Fig.3.12 Photograph of the 1.5% agarose gel stained with ethidium bromide showing product specific PCR obtained with DNA extracted from kidney samples of dog naturally infected with leptospire species. Lane M= molecular weight marker 1kb DNA ladder, Lanes 6 and 8 positive kidney sample of dogs.

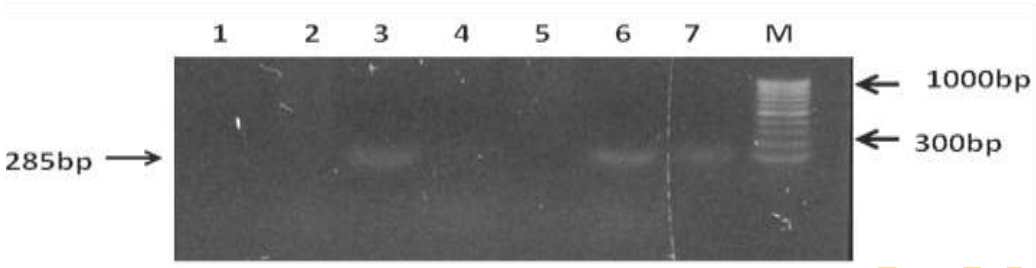

Fig.3.13 Photograph of the 1.5% agarose gel stained with ethidium bromide showing product specific PCR obtained with DNA extracted from kidney and blood sample of dogs naturally infected with leptospire species. Lane M= molecular weight marker 1kb DNA ladder, Lanes 3, 6 and 7 positive kidney sample of dogs.

3.3.11 Microscopic agglutination test using leptospiral monoclonal antibodies

Table 3.12 depicts different isolates of leptospiral organism form the kidney and blood samples of infected dogs. Thirty-four isolates from kidney samples and 3 isolates from blood samples were cultured but 27 were used in this study, because of contamination of the remaining 10 samples.

Out of the 27 isolates used, 3 were cultured from the blood of 3 dogs during acute infection while the remaining 24 were isolated from postmortem kidney samples of dogs. Out of 27 isolates, *L. icterohaemorrhagiae* had the highest prevalence in 8 (29.7%, 95%CI from 13% to 47%) dogs with agglutination titres of 1:1600. This was followed by *L. pomona* with 5 (18.5%, 95%CI from 4% to 34%) dogs and agglutination titres of 1:3200. *L. grippotyphosa* and *L. canicola* was 4 (14.8%, 95%CI from 1% – 29%) dogs each, but with different agglutination titres (1:3200 and 1: 800 respectively). *L. bratislava* was serotyped in 3 dogs (11.1%, 95%CI from 0.0 – 23%) with agglutination titres of 1: 1600. Out of the three leptospires cultured from the blood of 3 dogs, 2 belong to serovar icterohaemorrhagiae and 1 undertermined. *Leptospira hardjo* was not found in this study, while 3 isolates were undetermined.

Table 3. 12 Characterization and prevalence of leptospiral Isolates using monoclonal antibodies in microscopic agglutination test (MAT) with 27 positive kidney samples of dogs

No	Serotypes	No. positive	Prevalence (%)	95%CI Lower - Upper	Highest agglutination titers
1	*L. Pomona*	5	18.5	04 – 34.0	1: 3200
2	*L. Grippotyphosa*	4	14.8	01 – 29.0	1: 3200
3	*L. Hardjo*	-	-	–	-
4	*L. Bratislava*	3	11.1	00 – 23.0	1: 1600
5	*L. Canicola*	4	14.8	01 – 29.0	1: 800
6	*L. Icterohaemorrhagiae*	8	29.7	13 – 47.0	1: 1600
7	*Undetermined serovars*	3	11.1	00 – 23.0	-

3.3.12 Renal histopathological changes in dogs with leptospiral infection

Histopathological details of all the 60 kidney sections of dogs are depicted in Table 3.13 below.

Leptospiral organisms were present within the tubules, and intact and degenerate leptospires were observed in the proteinaceous tubular and glomerular casts (Fig.3.16-17). In all cases (both leptospire positive and negative tissues), there were histopathological alterations in the renal tissues.

Microscopically, 34 (77%, 95%CI from 67 – 91%) out of 43 leptospire positive kidney tissues showed severe interstitial nephritis characterized by severe multifocal to coalescing peritubular, perivascular and periglomerular lymphoplasmacytic inflammatory foci (Figs.3.13-14). But 10 (59%, 95%CI from36 – 82%) out of the 17 leptospire negative tissues showed mild interstitial nephritis with 9 (53%, 95%CI from 29 – 77%) and 7 (41%, 95%CI from 18 – 64%) showing perivascular and periglomerular lymphoplasmacytic cellular infiltration respectively.

Glomerulonephritis was observed in 8 (18.6%, 95%CI from 7 – 31%) out of the 43 leptospire positive tissues, characterized by 4 (9.3%, 95%CI from 0 – 18%) severe membranous glomerulonephritis, 2 (5%, 95%CI from 0-11%) mild membranoproliferative glomerulonephritis and 2 (5%, 95%CI from 0 – 11%) moderate embolic glomerulonephritis. Embolic glomerulonephritis was more prominent and severe in the leptospiral negative kidney tissues with 7 cases (41%, 95%CI from 18 – 64%). The membranous glomerulonephritis was more prominent in the leptospiral positive kidney section as demonstrated by the Periodic Acid Schiff stain with acellular glomeruli, thickening and splitting of the basement membrane (Fig.3.15).

Thirty-three (76%, 95%CI from 64 – 90%) out of the 43 leptospire positive kidney samples showed prominent or severe diffuse tubular nephrosis and 8 (19%, 95%CI from 07 – 31%)

showed moderate vacuolar degeneration of the tubular epithelial cells compared with 16 (94%, 95%CI from 83 – 100%) and 4 (24%, 95%CI from 4 – 44%) severe tubular nephrosis and mild vacuolar degeneration in the leptospiral negative tissues respectively.

Seventeen (39.5%, 95%CI from 25 – 55%) and 14 cases (33%, 95%CI from19 – 47%) of moderate to severe tubular and glomerular hyaline casts (respectively) were observed in the leptospiral positive tissues compared with 11 cases (65%, 95%CI from 42 – 88%) of tubular hyaline cast and 5 (29%, 95%CI from 7 – 51%) cases of glomerular hyaline cast in the leptospiral negative tissues. Tubular dilatation was more prominent in the leptospiral negative with 9 cases (53%, 95%CI from 29 – 77%) compared with 5 cases (12% 95%CI from 2 – 22%) in the leptospiral positive tissues. Mild to moderate interstitial oedema, interstitial fibrosis, renal haemorrhages, tubular haemochromatosis and pyelonephritis were also prominent lesions in both leptospire positive and negative kidney tissues. Other lesions such as tubular calcification, renal cysts, crystal formation and bacterial colonies were more prominent and conspicuous in the leptospire negative kidney tissues than in leptospire positive tissues. In all, renal congestion, tubular nephrosis, interstitial lymphoplasmacytic cellular infiltration, membraneous glomerulonephritis, hyaline casts and tubular dilatation were the most prominent and severe lesions in the leptospiral positive kidneys. Conversely, tubular nephrosis was the only most conspicuos lesions in the leptospiral negative kidney samples.

Table 3. 13 Histopathological changes in sixty kidneys of dogs with positive and negative leptospiral organism.

Lesions	**Lepto +ve** (n=43)	**%** (71.7%)	**95%CI** **Lower**	**Upper**	**Lepto -ve** (n= 17)	**%** (28.3%)	**95%CI** **Lower**	**Upper**
Renal congestion	10	23.3	10	36	10	59	36	82
Interstitial oedema	9	20.9	09	33	6	35	12	58
Tubular nephrosis	33	76.7	64	90	16	94	83	100
Tubular vacuolar degeneration	8	19	07	31	4	24	4	44
Interstitial fibrosis	7	16.3	05	27	8	47	23	71
Tubular haemochromatosis	2	5	01	11	1	6	00	17
Interstitial lymphoplasmacytic infiltration	34	77	67	91	10	59	36	82
Perivascular lymphoplasmacytic infiltration	38	88.4	78	98	9	53	29	77
Periglomerular lymphoplasmacytic infiltration	32	74	61	87	7	41	18	64
Membranoproliferative glomerulonephritis	2	5.0	0	11	2	12	0	28
Membranous glomerulonephritis	4	9.3	0	18	2	12	0	28
Embolic glomerulonephritis	2	5.0	0	11	7	41	18	64
Pyelonephritis	3	7.0	0	15	3	18	0	36
Glomerular casts	14	33	19	47	5	29	7	51
Tubular hyaline casts	17	40	25	55	11	65	42	88
Tubular calcification	2	4.7	0	11	4	24	4	44
Thickened renal capsule	1	2.3	0	7	1	6	0	17
Tubular dilatation	5	12	2	22	9	53	29	77
Renal haemorrhages	1	2.3	0	7	3	18	0	36
Renal cysts	-	-	-		4	24	4	44
Bacterial colonies	2	4.7	0	11	5	29	7	51
Crystals (type)	2	4.7	0	11	4	24	4	44

Lepto +ve = leptospiral positive, lepto-ve = leptospiral negative,

There was a significant increase ($p<0.05$) in the renal histopathological lesions in dogs with suspected leptospirosis compared with those that were negative (10.90±2.71 and 6.0±0.84 respectively) and showed positive correlation ($r = 0.70$, with a P value of 0.0002). Out of the 60 kidney sections examined using WSSS, 43 (71.1%) showed the presence of leptospire organism either as intact or granular leptospiral organism within the tubules.

Immunohistochemical staining revealed mild to moderate leptospiral antigens in the selected kidneys. Immunoreactivity was not limited to the tubular lumina but also in the interstitium Out of the 10 kidney samples examined immunohistochemically, only 7 (70%) showed mild to moderate immunoreactivity to *Leptospira icterohaemorrhagiae* antigens. Small and large granules were observed in the cytoplasm of tubular epithelial cells, (Figs.3.19-22) macrophages (fig.3.33) and endothelial cells. Cases that were negative by silver stain also were not immunoreactive to any of the 6 anti-leptospiral monoclonal antibodies used in this study, but not all cases that were silver stain-positive were immunoreactive to the monoclonal antibodies

Table 3. 14 Semiquantification of the histopathological changes in leptospiral positive and negative kidneys of dogs.

	Severity of the lesions	
Lesions	Lepto +ve	Lepto –ve
Renal congestion	+++	+
Interstitial oedema	++	+
Tubular nephrosis	+++	+++
Tubular vacuolar degeneration	++	+
Interstitial fibrosis	++	+
Tubular haemochromatosis	+	+
Interstitial lymphoplasmacytic infiltration	+++	+
Perivascular lymphoplasmacytic infiltration	+++	+
Periglomerular lymphoplasmacytic infiltration	+++	+
Membranoproliferative glomerulonephritis	+	++
Membranous glomerulonephritis	+++	+
Embolic glomerulonephritis	+	++
Pyelonephritis	+	++
Glomerular casts	+++	++
Tubular hyaline casts	+++	++
Tubular calcification	+	+
Thickened renal capsule	++	+
Tubular dilatation	+++	++
Renal haemorrhages	++	-
Renal cysts	-	++
Bacterial colonies	++	++
Crystals	+	++

Lepto +ve = leptospiral positive, lepto-ve = leptospiral negative, - = absent, + = mild, ++ = moderate, +++ = severe/marked.

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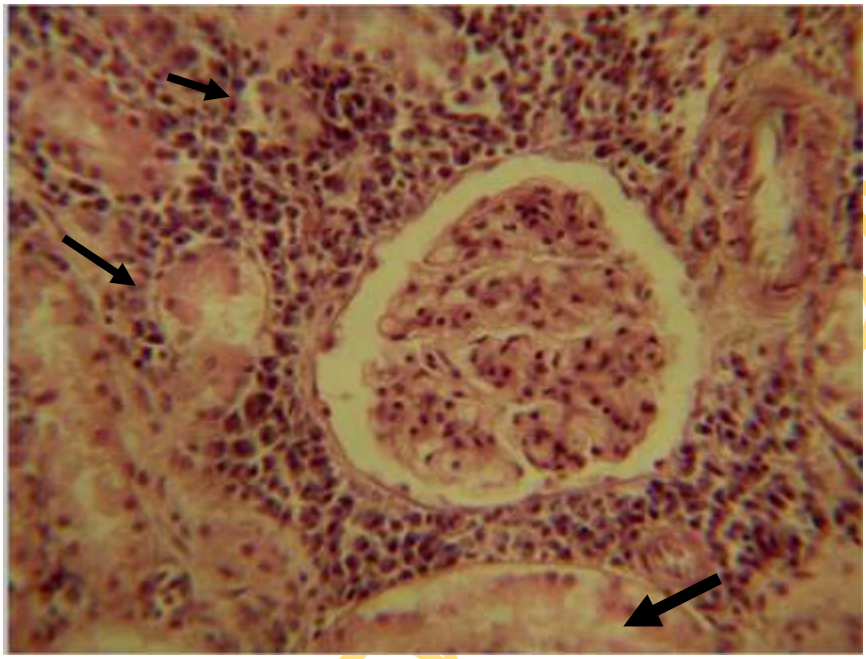

Fig. 3.14 Photomicrograph of the kidney section of leptospiral positive dog showing moderate, diffuse tubular degeneration and necrosis (big arrow) and atrophy (small arrows), with interstitial, perivascular and periglomerular lymphoplasmacytic cellular infiltration. H&E X 400.

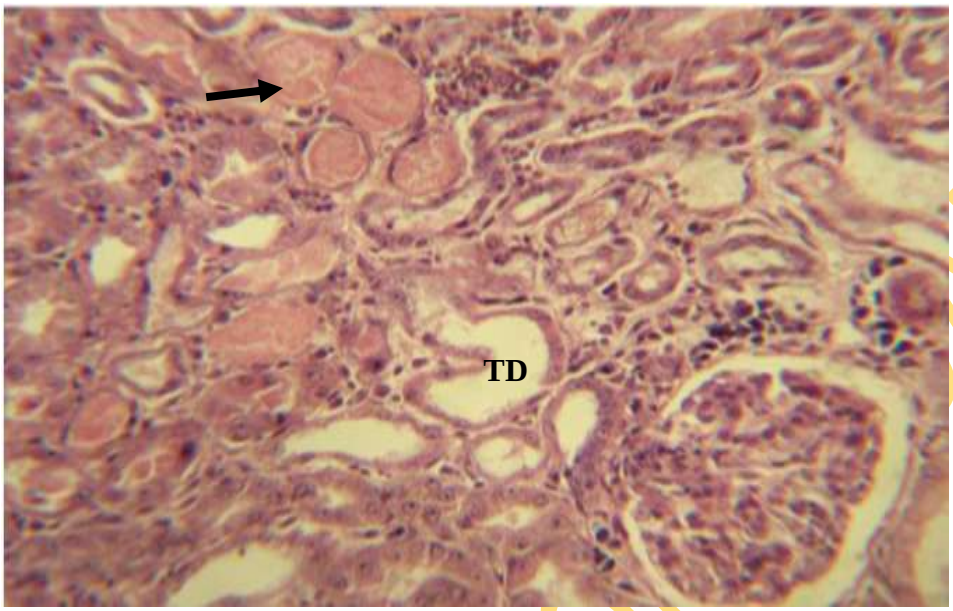

Fig.3. 15 Photomicrograph of the kidney section positive with leptospiral infection showing severe tubular degeneration and necrosis (arrow) and moderate tubular dilatation (TD) with diffuse but sparse mononuclear cellular infiltration. H & E x 300.

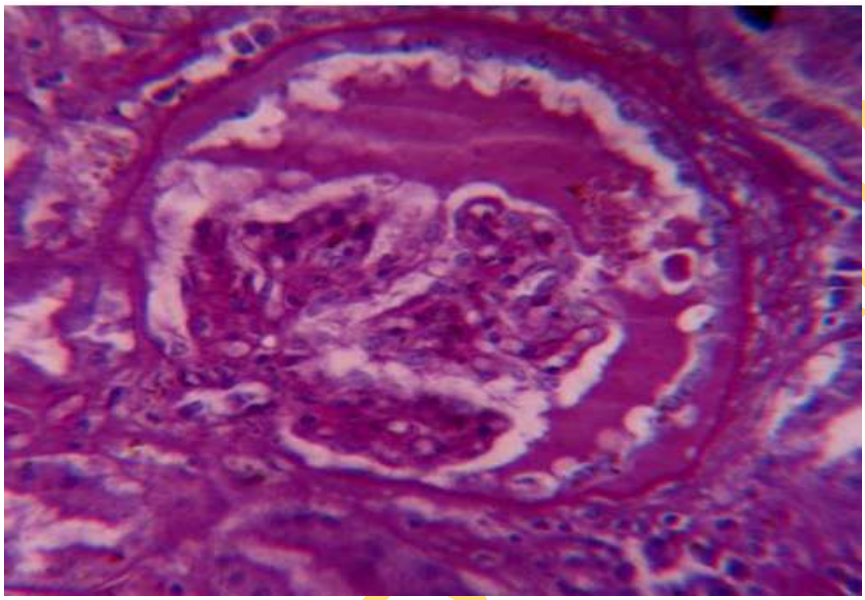

Fig.3. 16 Photomicrograph of the kidney section positive with leptospiral infection showing a membranous glomerulonephritis with hypocellular glomerular tuft, thickening and splitting of the Bowman's capsule and abundant protein cast in the Bowman's space. Periodic Acid Schiff. x 450

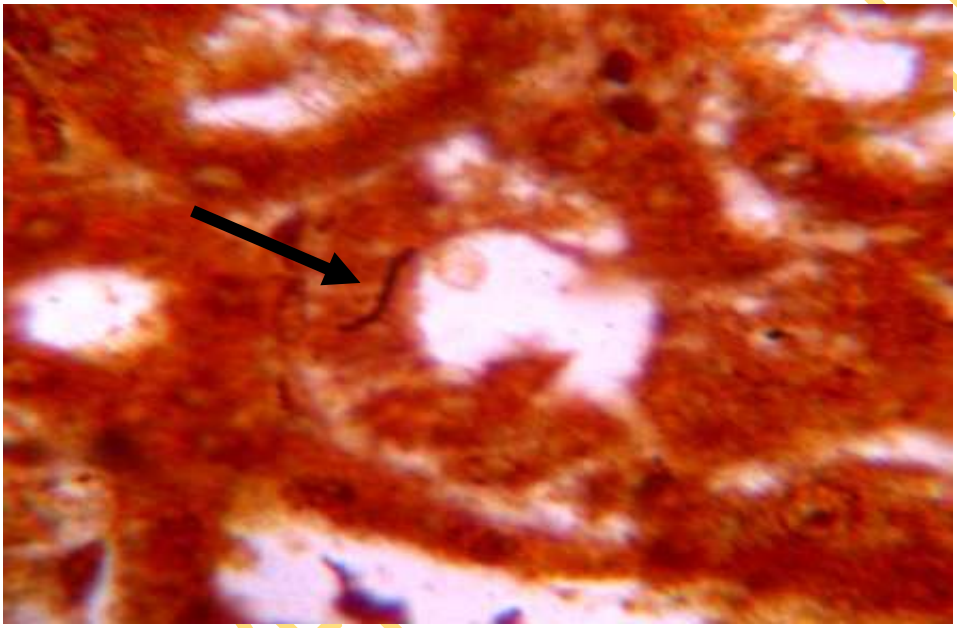

Fig.3. 17 Photomicrograph of the kidney section showing a leptospiral organism within the cytoplasm of epithelial cells (arrow). Warthin Starry silver stain. x 400

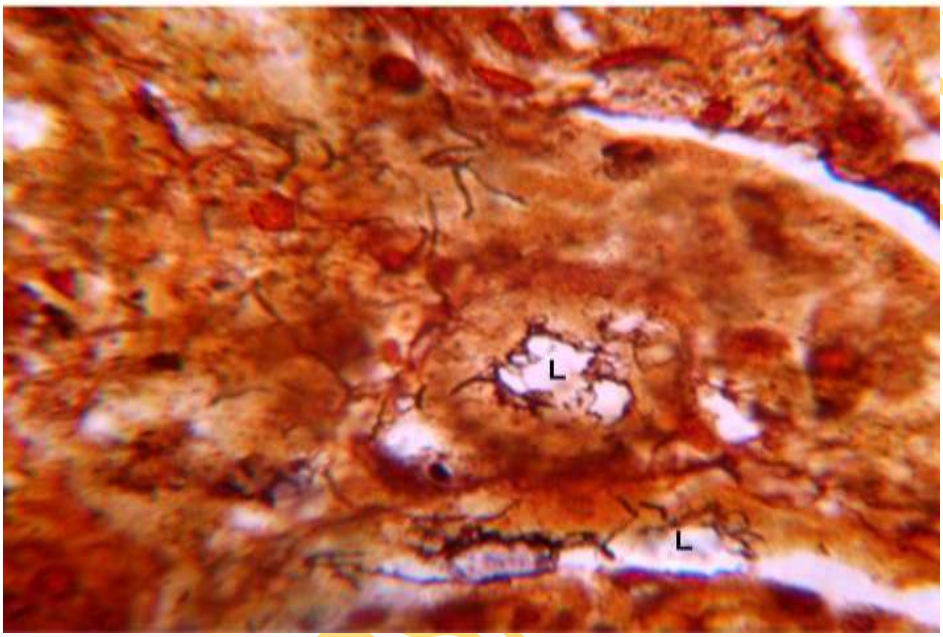

Fig.3.18 Photomicrograph of the kidney section showing severe Leptospira organism in the tubular lumina (L) and the cytoplasm of the epithelial cells. Warthin Starry silver stain. x 350

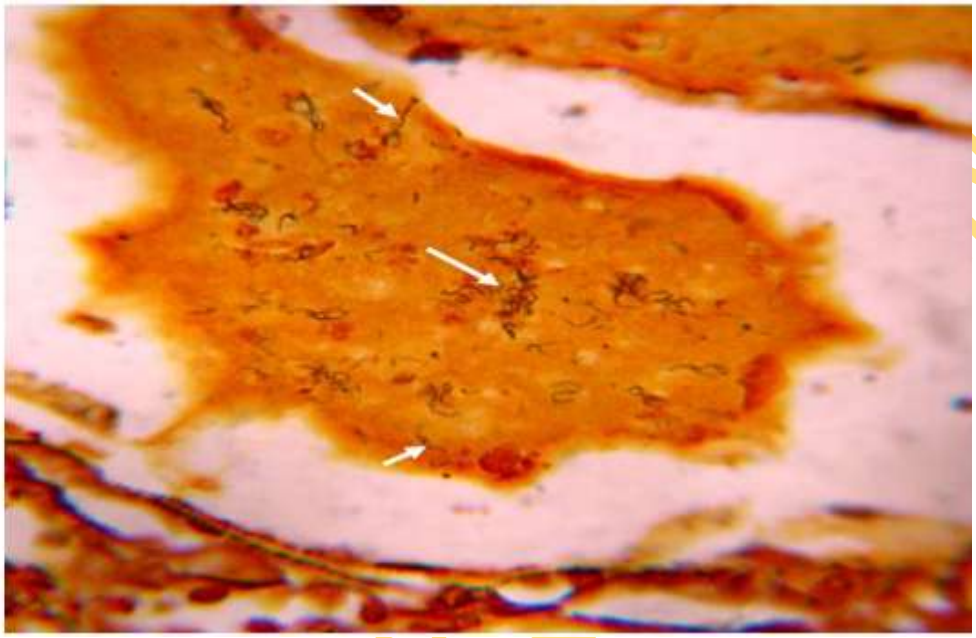

Fig.3. 19 Photomicrograph of the kidney section showing numerous leptospiral organisms in the protein cast (white arrows) within severely dilated glomerular space. Warthin Starry x 300

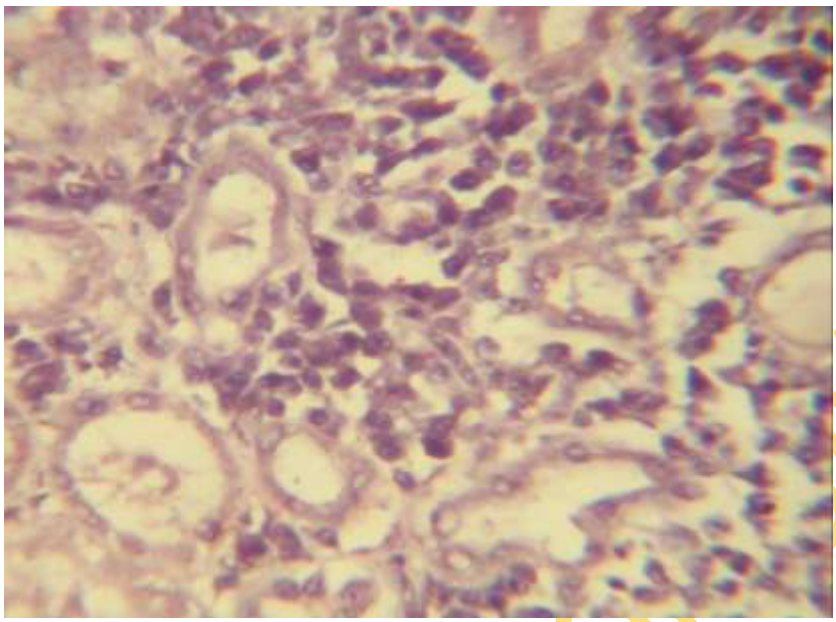

Fig.3.20 Photomicrograph of the negative-immunoreactive kidney section. Alkaline phosphatase-fast red counterstained with heamatoxylin stain. x 350

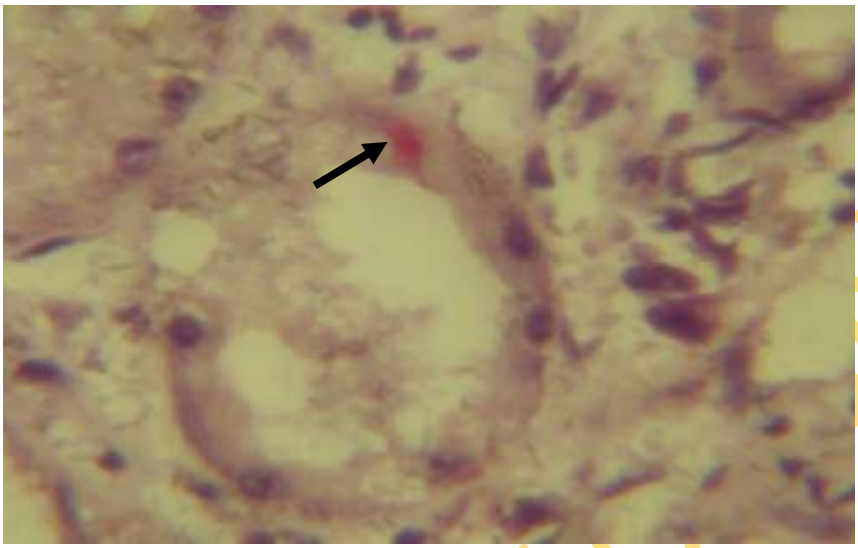

Fig. 3. 21 Photomicrograph of the kidney section showing immunoreactivity to *Leptospira interrogans* serovar *icterohaemorrhagiae* antigen in the cytoplasm of the epithelial cell (arrow) of the proximal convoluted tubule. Alkaline phosphatase-fast red, counterstained with heamatoxylin x 350.

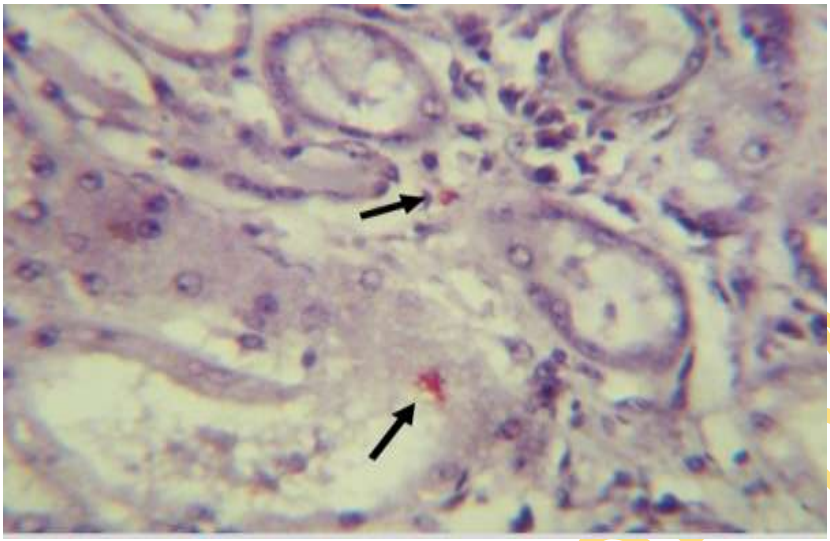

Fig.3. 22 Photomicrograph of the kidney section showing (A) immunoreactivity of *leptospira interrogans* serovar *icterohaemorrhagiae* antigen in both tubular lumen and the interstitium (arrows). Alkaline phosphatase-fast red counterstained with heamatoxylin x 300

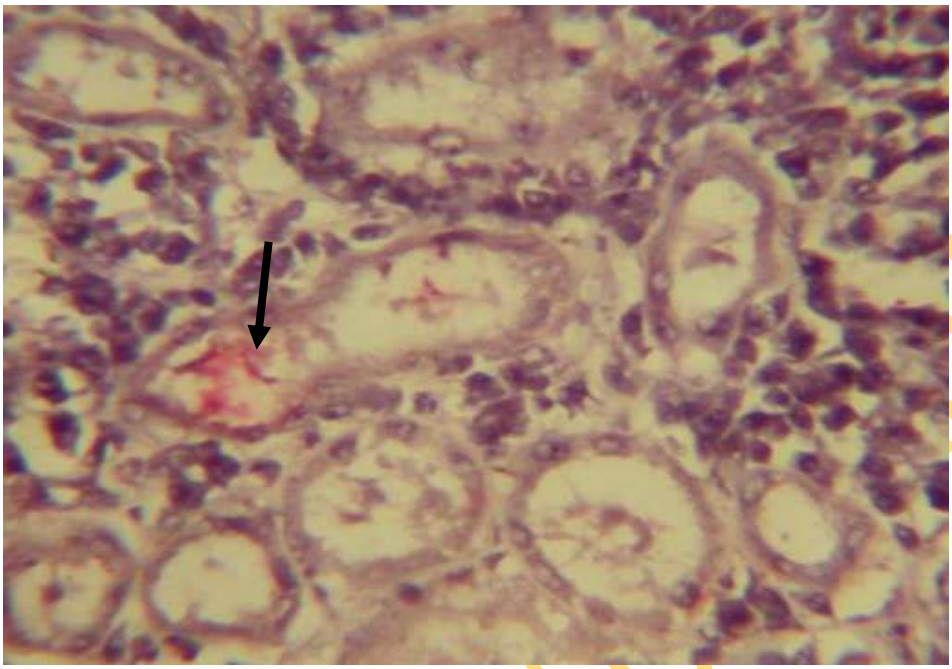

Fig.3. 23 Photomicrograph of the kidney section dog showing immunoreactivity of *Leptospira interrogans* serovar *icterohaemorrhagiae* antigen in tubular lumen (arrow). Alkaline phosphatase-fast red counterstained with heamatoxylin, x 300.

Table 3.15 Relationship between the degree of microscopic lesions (H & E) and the presence of leptospiral antigens (WSSS) observed in kidney sections of 60 dogs examined

	The intensity of leptospiral antigens			
.	-	+	++	+++
Degree of lesions				
-	1	2	-	-
+	4	6	3	2
++	11	4	5	4
+++	1	3	4	10
	17	15	12	16

- = absent, + = mild, ++ = moderate, +++ = severe/marked.

Out of the 60 kidney samples stainned with WSSS, only 3 were without histological lesions. Lesions were observed in 41 (95.4%) out of the 43 that demonstrated leptospiral antigens in the tubular lumina, compared with 16 (94.1%) out of 17 without the presence of leptospiral organism. There was significant difference ($P<0.05$) in the proportion of dogs having kidney lesions among infected and non-infected dogs.

3.3.13 Distribution and localization of leptospiral organism in the renal parenchyma: A hallmark of either acute or chronic infection in dogs

3.3.13.1 Leptospiral distribution and localization in acute infection

Out of the 43 kidney sections that were positive to leptospiral organism using Warthin Starry silver stain in this study, 20 (46.5%) of these kidneys met two or three criteria in section 3.2.11 suggesting acute form of leptospirosis in these dogs before they died. Table 3.16 showed the distribution and localization of leptospire organism during acute infection in the dog.

3.3.13.2 Leptospiral distribution and localization in chronic infection:

Table 3.17 showed the distribution and localization of leptospire organism during chronic infection in the dog. Out of the 23 kidney sections examined 21 (91.3%) showed severe degeneration and necrosis of the tubular epithelial cells, while 22 (95.7%) demonstrated mild to moderate leptospiral colonies closely or loosely attached to the apical surface of the tubular epithelial cells. Nineteen (82.6%) showed the presence of moderate to marked multiple foci of interstitial lymphoplasmacytic cellular infiltration. Evidence of regeneration such as cytomegaly and binucleation were observed in 8 (34.8%) of the sections examined.

Table 3. 16 Localization of leptospires organism in the renal parenchyma during acute infection in 20 dogs using Warthin Starry silver stain and Immunohistochemistry

No	Location of leptospiral organism/other factors	No	%	95%CI Lower - Upper
1	Within interstitium	2	10	00 - 23
2	Interstitium via basement membrane into the tubular epithelial cell's cytoplasm	2	10	00 - 23
3	Bowman's space and within tubular hyaline cast	4	20	02 - 38
4	Within cytoplasm or between adjunct epithelial cells	12	60	38 - 82
5	Presence of leptospires strand across degenerated epithelial cell's cytoplasm and the tubular lumen.	15	75	50 - 95
6	Absence of lymphoplasmacytic cellular infiltration.	19	95	85 - 100

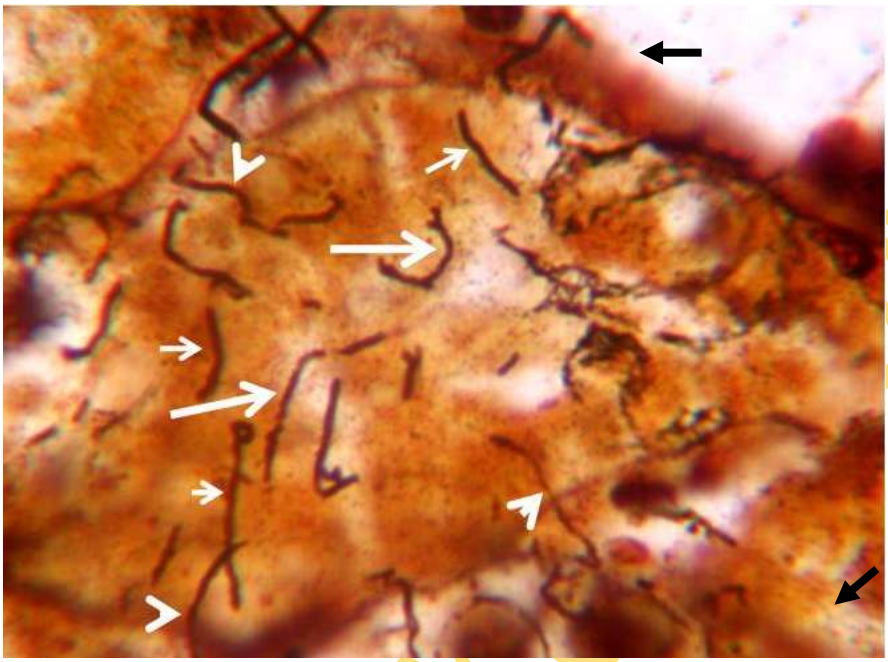

Fig.3. 24 Photomicrograph of kidney section showing leptospiral organism at different stages of tubular invasion in a dog with acute leptospirosis. Leptospires within interstitium (black arrows), from interstitium via the basement membrane into the tubular cytoplasm (white head arrows), leptospires within cytoplasm of epithelial cell (small white arrows) and leptospires strands across degenerated epithelial cell's cytoplasm and the tubular lumina (big white arrows) X500 Warthin Starry silver stain.

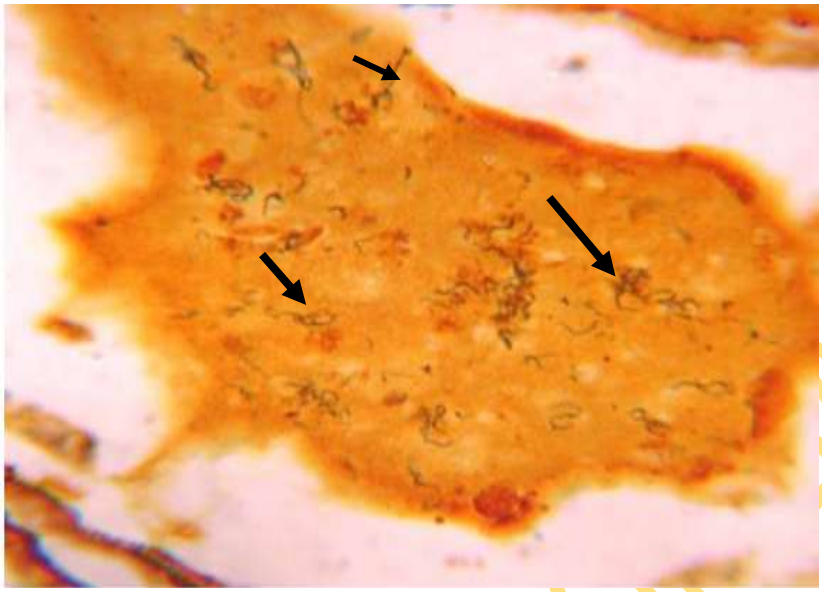

Fig.5. 25. Photomicrograph of kidney section showing numerous leptospiral organisms within proteinaceous cast (arrows) in severely dilated glomerular space of a dog. X 300. Warthin Starry Silver Stain.

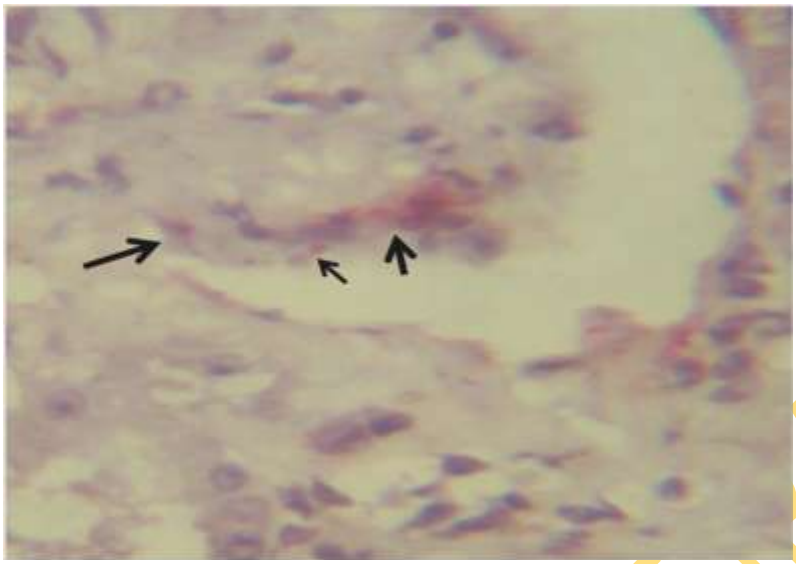

Fig.3.26 Photomicrograph of kidney section showing immunohistochemical demonstration of leptospiral antigen within the glomerulus (arrows). Alkaline phosphatase-fast red counterstained with heamatoxylin. x 350

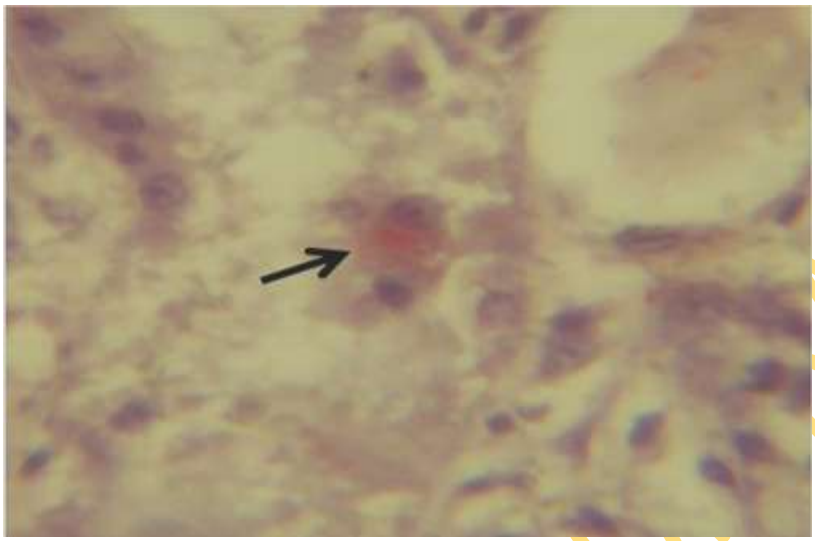

Fig.3.27 Photomicrograph of kidney section showing immunohistochemical demonstration of leptospiral antigen within the cytoplasm of the degenerated tubular epithelial cell (arrow) of a dog. Alkaline phosphatase-fast red counterstained with heamatoxylin, x 350.

Table 3.17 Localization of leptospiral organism in the renal parenchyma in chronic infection from 23 dogs using Warthin Starry silver stain and immunohistochemistry

No	Location of leptospiral organism	No	%	95%CI Lower-upper
1	Evidence of regeneration tubular epithelial cells such as cytomegaly and binucleation.	8	34.8	15 - 55
2	Presence of lymphoplasmacytic cellular infiltration	9		
3	Absence of lymphoplasmacytic cellular infiltration	14		
4	Severe degeneration of tubular epithelial cells	21	91.3	79 - 100
5	Leptospire colonies closely attached to the tubular epithelial cells within the lumen	23	95.7	88 – 100

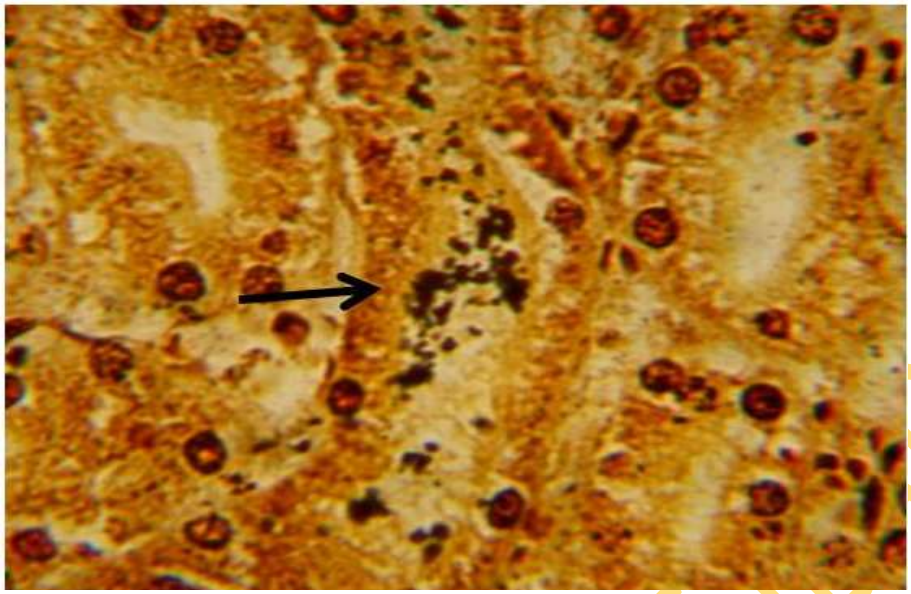

Fig.3.28 Photomicrograph of kidney section showing the presence of leptospiral granules (A) and organisms. Note: Absence of mononuclear cells infiltration within the interstitium. Warthin Starry silver stain. x 400.

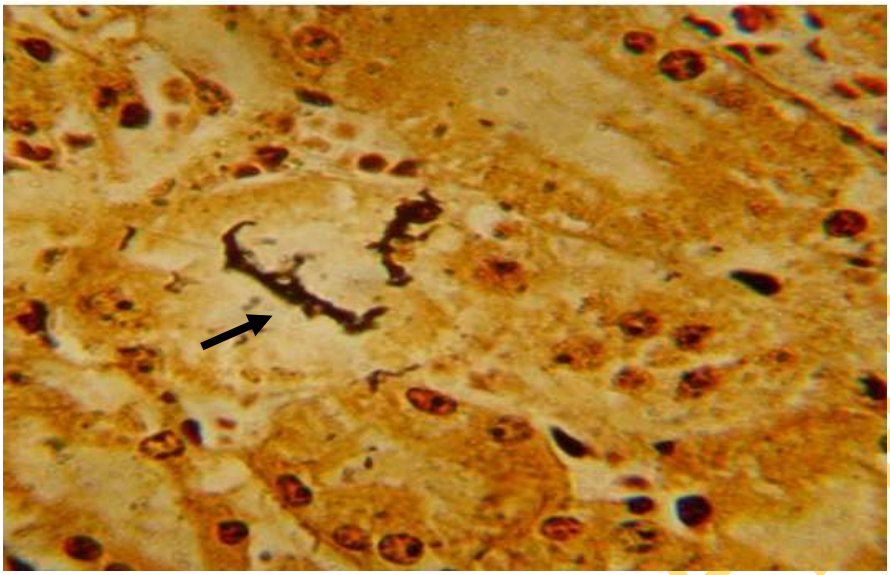

Fig.3.29 Photomicrograph of kidney section showing the presence of leptospiral organisms within the lumen of the proximal tubules (arrow) with degenerated epithelial cells. Note: Absence of mononuclear cells infiltration within the interstitium. Warthin Starry silver stain. x 400.

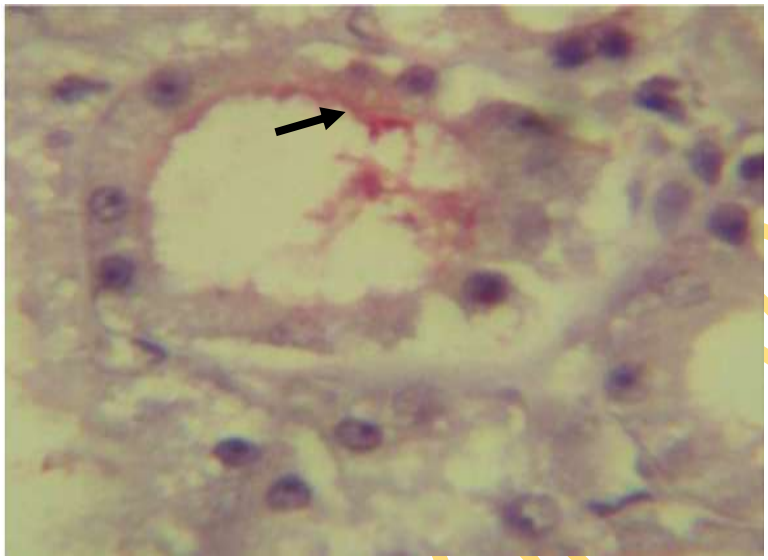

Fig.3. 30 Photomicrograph of kidney section showing the presence of leptospiral antigen within the lumen of the distal tubule closely attached to degenerated tubular epithelial cells (arrow) Alkaline phosphatase-fast red counterstained with heamatoxylin. x 400.

Table 3. 18 Comparative results of the four diagnostic techniques for the detection of leptospiral organism in organs of infected Dogs

Organs	EMJH/(44)	WSSS/ (60)	IH/(10)	PCR/(16)	MAT/(27)	Lesions/H.E
Liver	-	3	1	-	-	++
Kidney	34	43	7	4	21	+
Lung	-	-	-	-	-	+
Heart	-	-	-	-	-	+
Pancreas	-	-	-	-	-	+
Spleen	-	-	-	-	-	+
Brain	-	-	-	-	-	+
Blood	3	ND	ND	1	3	-
Total	37(84.1%)	46 (76.7%)	8 (80%)	5 (31.3%)	24 (88.9%)	.

3.4. DISCUSSION

This study has shown that the animal incidence of canine leptospirosis during the period of this study varied slightly. Yearly flunctuations in the incidence might be attributed to differences in other factors (e.g. the amount of yearly rainfall, flooding, outdoor activities and contact of dogs with reservoir host, most especially rats) and vaccination status of dogs against some of the isolated serovars in this study as suggested by Adesiyun *et al.* (2006). It is also possible that the increasing trend from 2003 to 2007 might have been reduced by vaccinations with appropriate serovars and the increasing awareness of possible sources of infection in the subsequent years. This might have led to the decrease in the incidence of suspected cases of canine leptospirosis from year 2008 to 2010. However, the yearly pattern of canine leptospiral incidence in this study might be bias by variability of submission of carcasses to these two Veterinary Teaching Hospitals, since as soon as a disease is recognized to be prevalent in a particular environment, there might be reduction in the number of dogs submitted for postmortem examination from different areas, thus giving a false impression or underestimation of the disease in that environment. Moreover, the well-known global warming effects in recent years and increasing rainfall in the study areas (Okewole and Ayoola 2009) might have also favoured or contributed significantly to the greater survival time of viable pathogenic leptospire in the environment since leptospires survive in a wide range of temperature (27-30^{0}C) and wet environment.

Sex and age have been identified as important factors in the epidemiology of canine leptospirosis (Miller *et al.*, 2007). Out of the 143 leptospiral suspected dogs according to sex in this study, male dogs (79, 44.4%) were more represented than the female dogs (64, 42.1%). This is in agreement with the report of other workers (Adesiyun *et al.*, 1997;

Seepersadsingh, 2003; Adesiyun *et al.*, 2006; Miller *et al*., 2007) but contrary to the report of Gautam *et al.* (2010) in which female dogs were more than the male dogs. This might not be unconnected with the fact that male dogs are kept as pets and guard dogs, making it necessary to have male dogs to watch over the homes as suggested by Adesiyun *et al.* (1997) and Seepersadsingh (2003). It is also possible that dog owners might want to avoid the menance of male dogs in their homes when the female dogs are on heat.

Different studies have identified age as an important risk factor for leptospirosis in dogs. Ward *et al.*, (2002) have suggested that dogs between 4 to 10 years of age were at increased risk of the disease, compared with dogs less than 1 year of age. In this study, dogs less than 1 year of age suspected of leptospirosis were moderately higher (59/147, 33.3%, 95%CI = 26% to 40%) compared with dogs above 4 years of age, being 30 out of 95 dogs (17%, 95%CI= 11% to 23%). This might suggest a combination of inadequate maternal antibody protection and immunological immaturity as suggested by Miller *et al*. (2007). This also stresses the significance of maternal immunity and the need to vaccinate dogs as early as 6 weeks of life as suggested by Adesiyun *et al.*, (2006). Dogs between 1-4 years of age had the highest prevalence in this study. This is in accordance with the report of other authors (Adin and Cowgill, 2000). They suggested that dogs are more prone to sniffing at this age group which increases the likelihood of exposure to contaminated urine. The lower percentage of dogs suspected of leptospirosis at ages greater than 4 years in this study is in agreement with the works of Gautam *et al.* (2010). Although, it is unknown whether this reduction in the disease risk at this age is attributable to lower exposure risk, possible previous vaccination or lower population of dogs at this age group. Niwetpathomwat and Assarasakorn (2007) have also suggested that dogs within the between age 1 to 4 years spend more time outside and might likely increase their exposure to leptospiral organism in the environment.

Thirteen different breeds of dogs were represented in this study. Incidence rates might possibly reflect the general dog population types in this part of the country, especially in the urban areas, but this may not be a true reflection of the breed of dog population in the rural areas where more Nigeria local dogs are found. The high incidence rates of leptospirosis in German shepherd dogs and other large breed of dogs in this study might not be unconnected with preference for security purpose in many homes. However, there is insufficient number of cases of other breeds of dogs to determine if some breeds were more resistant than others.

Clinical signs observed in this study were retrieved from case records and might possibly be dependent on completion of accession forms by the clinicians on duty as at the time of case presentation. While it is reasonable to assume that the salient clinical signs would be recorded, it is also possible that forms were incompletely filled in for various reasons as suggested by Miller *et al.* (2007). Recently, Goldstein *et al*. (2006) suggested that infection with different serogroups only causes minor differences in clinical symptoms. The most common clinical presentations of leptospirosis in dogs in this study were nonspecific signs compared with leptospiral negative dogs. These include; pyrexia (n=84), anorexia (n=82), lethargy or depression (n=46), vomiting (n=28) and abdominal pain (n=6) which are findings that are similar to those in other reports of canine leptospirosis (Prescott, 2002; Miller *et al.*, 2007). These clinical signs might be a reflection of multisystemic organ failure rather than a specific organ disease. The abdominal pain with the haematuria might suggest acute renal infection as suggested by Ward *et al*., (2004). Clinical signs such as haemoptysis, haematuria, epistasis and haematochezia might have been due to vascular diathesis as was observed in this study.

In canine leptospirosis, the presenting clinical signs depend on the severity and duration of infection (Miller *et al*., 2007), and these clinical manifestation have been shown to be nonspecific, but signs such as lethargy, anorexia and jaundice have been reported to be more

prevalent in clinical presentation of the disease in dogs in the literature (Prescott *et al*., 2002; Miller *et al.,* 2007). This is not in agreement with the observations in this study in which pyrexia was the most prevalent clinical manifestations.

It is well understood that the results obtained in this study might have been bias towards dogs with more severe icterus and without ticks on the body. At the same time, dogs infected with subclinical leptospirosis and showing few or no typical clinical signs might have been omitted during the course of this investigation. Thus, it is likely that the results presented in this study might have been biased towards more severely affected cases rather than being representative of all canine leptospirosis in the studied area.

The most common laboratory abnormalities encountered in dogs with acute leptospirosis are anemia, leukocytosis, azotemia, electrolyte disturbances, mild to moderate increased liver enzymes (ALP, ALT or AST), isosthenuria and proteinuria (Miller *et al.,* 2007). In the present study, low PCV, low Hb, and low RBC indicated severe aneamia in the 122 dogs and this is characterized by nonresponsive microcytic normochromic and normocytic normochromic anaemia. Anaemia has been documented in the literature in cases of canine leptospirosis (Prescott *et al.,* 2002; Miller *et al.,* 2007) but the pathogenesis of this anaemia still remain elusive. However, it is possible to suggest pathways or the mechanisms of these types of anaemia in two folds depending on the duration of the condition: haemorrhagic diathesis (i.e. the loss of blood inform of petechial, ecchymotic, haematochexia, and epistaxis) in different organs might have resulted into iron deficiency anaemia and consequently microcytic normochromic anaemia observed in some dogs with severe vascular disturbances in this study. Secondly, leptospiral invasion and destruction of tubular epithelial cells including juxtaglomerular apparatus, that secret erythropoietin might have probably cause decrease in erythropoietin production and consequently led to erythropoietin deficiency to stimulate different stages of erythropoiesis or the direct effects of toxin produce by

leptospire (haemolysin) on the erythroid progenitor cells in the bone marrow. This might have led to the normocytic normochromic type of aneamia observed in this study. This is consistent with the works of other authors (Birnbaum *et al*., 1998; Miller *et al*., 2007) in the literature.

Leukocytosis due to neutrophilia with left shift has been a consistent finding in leptospirosis in the literature (Edwards *et al*., 1982; Levett, 2001). Miller *et al*., (2007) documented stress leukogram in 18 dogs with leptospire infection examined in Australia. In this study, leukocytosis due to neutrophilia with left shift was observed in the some dogs, despite the fact that the mean WBC of the dogs were within the normal upper limit but the high level of immature cells suggest a possible regenerative or degenerative left shift in these dogs.

The significant monocytosis observed might have been due to tissue demand, which is consistent with the report of other workers (Prescott *et al*., 2002; Miller *et al*., 2007).

There are conflicting reports in the literature on the occurrence of thrombocytopenia observed in leptospirosis infection. Some authors were of the opinion that it is due to disseminated intravascular coagulopathy (DIC) while some were of the contrary opinion (Yang *et al*., 2006). In this study, although there was thrombocytopenia, but the low platelet level might not have been sufficient to cause DIC in the affected dogs (Table 3.6).

The gross and histopathological changes observed in various organs such as the pancreas, lung, brain, spleen liver and heart in this study are consistent with studies elsewhere (Langston and Heuter, 2003). Although, leptospiral organisms are extensively disseminated to almost all the tissues and organs during the early stages of infection (Athanazio *et al*., 2008), but leptospires were not detected in many of these organs (except kidney and liver) by the various methodologies used in this study. This might be as a result of clearance of the

organism from most of the tissues by circulating anti-leptospiral immunoglobulins which are produced at the earlier phase of the infection as suggested by Monahan *et al.*, (2008).

The possibility of identifying all the infecting serovars of leptospiral organism in all the dog samples that demonstrated leptospire antigen with WSSS was not possible in this study. This might be due to the fact that not all kidney samples were cultured and those that were not positive to any of the six monoclonal antibodies might belong to other serogroup different from those used in this study.

However, the rabbit monoclonal antibody RGA specific for *leptospira interrogans* serovar *icterohaemorrhagiae* showed immunoreactivity in most of the kidney, while others were negative on the same kidney samples.

The presence of leptospire antigens in the tubular lumina, tubular epithelial cells and within the cytoplasm of interstitial macrophages (figs.3.32-35) in granular form is in agreement with the reports of other investigators (Wild *et al.*, 2002). This is not in agreement with the reports of some investigators, who used immunoflourescent and immunoperoxidase techniques (Morrison and Wright, 1976; Pereira *et al.*, 1997; Barnett *et al.*, 1999); in which there was loss of tissue structural architecture which consequently made it difficult in assessment and evaluation of the associated pathological changes and the location of the antigen in the renal tissue.

The cellular infiltrate involved in the interstitial nephritis associated with leptospiral infection is made up of lymphocytes, macrophages, plasma cells and occasionally neutrophils (Sitprija *et al.*, 1980; Barnett *et al.*, 1999; Wild *et al.*, 2002). Plasma cells infiltrates in the kidneys of dogs with leptospirosis has been confirmed by immunohistochemistry to contain and secret local anti-leptospiral antibodies (Wild *et al.*, 2002). Wild *et al.*, (2002) demonstrated the presence of IgG in 65% and IgM in 35% of plasma cells in the kidney of dogs with leptospiral infection. Although, IgG and IgM leptospira antibodies were not determined in

this investigation, but 95% of the cellular infiltrate in most of the leptospire positive kidneys observed were plasma cells (figs.3.24-6). The immunoreactivity of leptospire antigens in the interstitial macrophages (Fig.3.33) interspersed within the plasma cell also demonstrated the possibility of plasma cells been activated by the antigen presenter cells (macrophages) to produce anti-leptospiral immunoglobulin.

The occurrence of glomerulonephritis has not been associated with leptospirosis in both animals and human infections. In this study, membraneous and membranoproliferative glomerulonephritis seemed to be more common in the leptospiral positive kidneys. These two glomerular histopathological alterations are usually associated with immune-mediated conditions (Maxie and Newman, 2006). In this investigation, the antileptospira antibody produced in the plasma cells in the kidney and other lymphoid tissues might have contributed to these pathological changes. It is also possible that myriad of environmental, biological, nutritional and chemical factors might induce such changes in the kidneys of dogs (Tucunduva de Faria *et al.,* 2007).

Knowledge of the prevalent leptospire serovars and their maintenance hosts is essential to understanding the epidemiology of the disease in any region (Levett, 2001). Over the years, leptospire icterohaemorrhagiae and canicola were thought to be the most prevalent serovars worldwide (Greene and Shotts, 1990). Most of the studies carried out in the literature were based on serological diagnosis and few works on the isolation and characterization of leptospire isolates in dogs. In the recent works of Okewole and Ayoola (2009) in the Southwestern Nigeria, the presence of other serovars (such as grippotyphosa, pomona and bratislava) were serologically documented in the dog population. In this study, isolation and characterization of leptospiral isolates were performed to establish the authenticity of these studies in the southwestern Nigeria. Serovars such as pomona (5 cases, 18.5%), gripptotyphosa (4 cases, 14.8%) and bratislava (3 cases, 11.1%) in addition to serovar

canicola (4 cases, 14.8%) and icterohaemorrhagiae (8 cases, 29.7%) were isolated from blood and postmortem tissues. The presence of 3 unidentified serovars with monoclonal antibodies and lack of immunoreactivity by the positive liver and kidney samples in this study, suggest that there are other unidentified serovars in the dog population in the studied area apart from grippotyphosa, pomona, canicola, icterohaemorrhagiae and bratislava isolated in this study. There is also the possibility that the 3 unidentified serovars might have been the emergence of new serovars which were not included in the monoclonal antibodies used. Moreso, the absence of leptospiral antigens in the various organs such as pancreas, heart, lung, spleen, lymph nodes and brain might have probably been due to (1) clearance of leptospiral organism from these organs before death by the immune system as suggested by Monahan *et al.* (2008) and Athanazio *et al.* (2008) or (2) due to the use of leptospirucidal antibiotics which might have cleared the organism from these organs before death.

Presently, there are no vaccines which contained all the 5 serovars isolated from the 41 dogs in this study. This shows that broadbased and all-encompassing vaccines should be developed to prevent further mortalities of dogs in Nigeria.

The mode of transmission of leptospires to dogs is unknown in this part of the country. It is likely to be associated with contact with the urine of both wild and domestic animals, notably rats, mice, goat, sheep, and cattle (Sullivan, 1974). This contact may be direct; for example, a house dog attacking a rat or indirect through contact with the contaminated environment such as water, soil and urine. In recent years, reports from several veterinary clinics (Okewole and Ayoola, 2009) and unpublished post mortem reports have indicated increase in the incidence of suspected canine leptospirosis in dogs appropriately vaccinated leading to speculations that either the two serogroups (canicola and icterohaemorrhagiae) contained in all the vaccines used locally are not responsible for most of the cases of canine leptospirosis in the country or that the vaccines were not potent due to improper storage temperatures.

It has been documented that different serovars may be responsible for leptospira infections, depending on the geographical location, thereby resulting in ineffective vaccination (Weekes *et al.*, 1997; Adin and Cowgill, 2000; Scanziani *et al*., 2002). For instance, in Turkey, the prior frequently detected serovars in canine leptospirosis were canicola, grippotyphosa and icterohaemorrhagiae until 2005 when Aslantas *et al.* (2005) reported bratislava as being predominant, demonstrating a significant change in the epidemiology of canine leptospirosis in that country. Recently, Okewole and Ayoola (2009) showed serological evidence of other serovars such as Grippotyphosa, Pomona and Bratislava in dogs in the studied areas of south west Nigeria. The characterization of different serovars of leptospiral organism (Grippotyphosa, Pomona and Bratislava Icterohaemorrhagiae and Canicola) in this study confirmed the findings of these workers.

It is therefore obvious from the studies on characterization and immunohistochemistry that *leptospire interrogans icterohaemorrhagiae* is the most prevalent serovar in the southwestern part of Nigeria. It is also possible from this study that the presence of serovar canicola, unlike serovar icterohaemorrhagiae, in all the vaccines used locally has virtually reduced or eliminated infection by this serovar.

Immunohistochemistry has been suggested as a promising diagnostic tool for leptospirosis in the kidney tissues especially formalin fixed tissues (Wild *et al.*, 2002). The serovars specific immunoreactivity of 7 (70%) out of 10 kidney sections and the detection of leptospiral antigens in 1 out of 3 liver sections shows that immunohistochemistry is an effective diagnostic tool to the serovar's level over the Warthing Starry silver stain. The absence of positive immunoreactivity in the remaining 3 kidney and 2 liver sections might be due to; 1) absence of leptospiral antigens in the small area of the kidney and liver sections examined, 2) the use of leptospirocidal antibiotics given prior to the death of the animals, and 3) insufficient leptospiral antigen in the renal tubules as suggested by Ross *et al*., (2011).

The use of polymerase chain reaction, culture isolation and microscopic agglutination test (MAT) in the diagnosis of natural canine leptospirosis in acute or chronic infection might not be sufficient to determining the duration or the progress of the disease as at the time of death in affected dog. Despite this challenge, the localization and distribution of leptospiral organism in the renal parenchyma might be a pointer to the stage or duration of the disease based on the documented studies in the literature. This can be made possible with diagnostic techniques such as Warthin Starry silver stain and immunohistochemistry. Based on the findings of different workers (Marshall, 1980; Sitpirija *et al.*, 1980; Cherille *et al.*, 1980; Yener and Keles, 2001; Tuncuduva de faria *et al.*, 2007) on the dissemination and invasion of leptospirosis in the renal parenchyma, it is possible to affirm from this study that out of the 43 kidney samples positive to Warthin Starry silver stain and immunohistochemistry, 20 dogs (46.5%) died of acute infection (Figs.3.36-39) while the remaining 23 (53.5%) died of leptospirosis at the chronic stage of the disease (Figs.3.40-41).

Although, the movement of leptospire from the capillary blood vessels via the endothelial cells into the interstitium has been demonstrated in the literature (Marshall, 1980), but the movement of the organism at different stages from the interstitium into the tubular lumina using special staining techniques such as WSSS and immunohistochemistry are rare findings in the literature.

CHAPTER FOUR

4.0 CULTURAL ISOLATION AND CHARACTERIZATION OF PATHOGENIC *LEPTOSPIRE SPP.* AND ASSOCIATED RENAL PATHOLOGY IN WILD RATS

4.1. INTRODUCTION

Over the years, house rats have been known to be asymptomatic chronic carrier of pathogenic leptospiral organism all over the world (Ido *et al*., 1917). They are the most important source of human infection wherever there is outbreak of leptospirosis in the urban settlement (Sarkar *et al*., 2002, Bharti *et al*., 2003). Earlier studies in rat models noted a marked natural resistance to lethal leptospiral infection which could be overcome by administration of ethionine and cyclophosphamide (Bertok *et al*., 1964; Thiermann, 1980). Recently, the *R. norvegicus* model was used to contrast the chronic, asymptomatic, leptospiral infection with the acute, lethal infection seen in the guinea pig model (Nally *et al*., 2005a). Mouse model has been used to investigate the role of specific genes in susceptibility (Nally *et al*., 2005b; Viriyakosol *et al*., 2006). However, mice are not an ideal model of resistance to acute disease as variations in strain, inoculum size and mouse age are known to affect outcomes (Faine, 1962).

Rats are an ideal model to study renal colonization since this process does not result in death or any notable histopathology (Tucunduva de faria *et al*., 2007). Faine, (1962) affirmed that the epithelium of the renal convoluted tubules apparently becomes resistant to reinvasion by leptospires present in the lumen soon after establishment of infection (Faine, 1962). A recent study observed antigenic switching of the leptospiral surface in host species with both susceptible guinea-pig and resistant rat in acute lethal disease (Nally *et al*., 2005a) and this might be one of the factors that enhance tubular colonization in rats. Various studies have shown that the duration of leptospiral carrier state or period depends on different leptospiral

serovars. Athanazio *et al.,* (2008), monitored chronically infected rats with *Leptospira interrogans* serovar *Copenhageni* strain Fiocruz L1-130 for 40 days. Thiermann (1981) also showed that rats infected with serogroup icterohaemorrhagiae strain maintained renal carrier state for 220 days compared with serogroup grippotyphosa strain for only 40 days.

Many factors such as population density, climate, pH, temperature and the degree of contact between maintenance and accidental host, have been shown to influence the extent to which infection is transmitted (Levett, 2001). The broad range of mammalian reservoirs explains a diverse array of epidemiological contexts such as rural endemics, urban outbreaks related to rainy seasons and floods, and emergent disease related to water sports and recreational exposure in developed countries (Tucunduva de faria *et al.*, 2007).

Different rodent species have been shown to be reservoir host of different serovars of leptospiral organism (Ido *et al.*, 1917; Alejandro 1977; Levett, 2001; Scanziani *et al.,* 2002; Priya *et al.,* 2007), but rats are generally maintenance host for serovars of the serogroups icterohaemorrhagiae and Ballum while mice are the maintenance host for serogroup Ballum (Levett, 2001).

In different parts of the world, especially Salvador, Brazil, leptospirosis occurs as epidemics related to the rainy season, floods and rat infestation of homes, with mortality frequently higher than 50% in humans (McBride *et al.,* 2005). In India, serology and isolation revealed that field rats are major natural carriers and shedders of leptospires in and around Madurai (Priya *et al.*, 2007). In epidemiological pattern of leptospirosis in the tropical wet areas, many serovars abounds which infects human and animals through large number of reservoir host, including rodents, farm animals and dogs which facilitate widespread environmental contamination (Faine, 1999). Despite the importance of the rat in disease transmission, few reports have focused on the isolation and characterization of leptospire serovars with the associated underlying pathology. Pathological studies of captured wild rats have shown a

broad spectrum of histological lesions which may not necessarily be related to leptospires (Laurain, 1955).

In Nigeria, over the past decade, based on postmortem reports many cases of supposedly vaccinated dogs have died as a result of leptospiral infection without knowing the source of infection and the serovar involved (Okewole and Ayoola, 2009).

Although in the works of Okewole and Ayoola (2009) different serovars of leptospiral organisms such as gripptothyphosa, Bratislava and Pomona were serologically detected from the sera of dogs but the source of infection are yet to be understood. Few works carried out in rats in Nigeria have been based primarily on serological evidence (Diallo and Denis, 1982) in the Northern parts of the country and no attempt has been made to isolate and characterized the prevailing serovars and the associated underlying renal histopathology in the rats population in the south western part of Nigeria.

The epidemiology of leptospirosis in any environment is best reflected by the serovars carried by the rodents and other domestic animals in that environment. Therefore, this present study was undertaken in order to; 1) determine the source of leptospiral infection to other domestic animals, especially dogs, 2) determine the prevalence of leptospiral infection in the wild rats population from three states, in South-western Nigeria, 3) characterize the various serovars in the wild rats and correlate them with the prevailing serovars in dogs 4) determine the effects of rat's age on the tubular localization and colonization of leptospire 5) determine a possible underlying renal pathology associated with leptospiral infection in the wild rats and 6) determine the best diagnostic method in the diagnosis of leptospirosis in the wild rats.

4.2 MATERIALS AND METHODS

4.2.1 Rat Capture protocols

Rats were trapped near the household of people in which their pets came down with leptospiral infection in three urban cities (Lagos, Ibadan and Abeokuta) of three states (Lagos, Oyo and Ogun) in south-western part of Nigeria. Rats were also trapped in areas where there were no reported cases of leptospiral infection in the same cities. Trapping was carried out for a period of 6 months, between November 2009 and April 2010. Traps (15cm x 25cm, Cash and Kill, China) were used in this study. The traps were placed close to dustbins, gutters and where their tracks and holes were identified within and outside the household. The captured periods were usually late in the evening and atimes overnight till the following morning. Over this period, 105 rats were trapped dead from three different cities. The rats were taken to the Department of Veterinary Pathology, University of Agriculture, Abeokuta within 2 to 10-hours of capture with the aid of ice packs for necropsy.

4.2.2 Rats Identification

The rats were identified according to the phenotypic characteristics described by Tucunduva de Faria *et al* (2008). Briefly, the rats were identified as *Rattus Rattus* based on the following physical features; a grey-brown coat with lighter ventral part, tail shorter than the combined head and body length. Younger rats were defined as 10 – 17cm with 50g – 300g and 18cm - 26cm for adults with 400g – 750g. Twelve rats (8 males and 4 females) were trapped from Lagos state, 44 rats (30 males and 14 females) from Abeokuta, Ogun-State and 49 rats (32 males and 17 females) from Ibadan, Oyo-State.

4.2.3 Sample collection

The ventral surface of the rats was disinfected with a good disinfectant (Methylated spirit) before opening of the abdomen with a pair of scissor. Kidney samples for culture isolation, polymerase chain reaction (PCR) and histopathology were collected within 3-4 hrs upon arrival from the place where the rats were trapped.

4.2.4 Bacteriological Studies

4.2.4.1 Preparation of EMJH medium

The Ellinghausen-McCullough-Johnson-Harris medium (EMJH) (Difco-USA) was used for leptospiral isolation modified with the addition of 10% rabbit serum enriched with calcium chloride (1%) and magnesium chloride (1%) (Alves, 1995). This culture medium was prepared with the addition of 5- fluorouracil (400mg/L; Sigma-USA) (Heer *et al.*, 1982), chloramphenicol (5mg/L; Sigma-USA), nalidixic acid (50mg/L; Inlab-BR), and neomycin (10mg/ L; Sigma-USA).

4.2.4.2 Kidney sample preparation and inoculation

Kidneys were rinsed with sterile distilled water and put in a sterile Petri dish. A small piece of 0.5 cm^3, containing the cortex and medulla was cut and macerated using sterile rat toothed forceps in the Petri-dish and then suspended in 0.5 ml PBS (pH 8.0). The suspension was then allowed to stay for 10min to allow the leptospira organism to flow out from the macerated kidney into the PBS.

Two to three drops of the PBS-macerated kidney samples were inoculated into 5ml of EMJH-FU medium. This was incubated at room temperature (28-30ºC) in the dark and examined under dark field illumination within 24 hours and intervals of 10 days to check for the growth of leptospires for at least three months.

4.2.5 Characterization of leptospiral isolates using Monoclonal Antibodies

The microscopic agglutination test using monoclonal antibodies in this study was according to the method of Obregón *et al*. (2007). Briefly, 50 µL of PBS was placed in each well from 2 – 6 of microtitre plate. Already prepared 50 µL monoclonal antibody at the dilution rate of 1:100 in PBS was placed in wells 1 and 2. Serial dilution of antibodies from well 2 to 6 were made in phosphate buffered saline (PBS) pH 7.2 in the microtitre plates. Then 50 µL aliquots of 7-10 days old culture of live leptospires in EMJH medium was added to each well. After 2-4 h of incubation at 37 ºC, the agglutination titre was determined by dark field microscopy. The whole set of Pomona, Bratislava, Hardjo, Grippotyphosa, Icterohaemorrhagiae and Canicola monoclonal antibodies were kindly offered by the Royal Tropical Institute, Amsterdam.

4.2.6 Detection of leptospiral DNA in the kidney of naturally infected rats using polymerase chain reaction

DNA was extracted from fully-grown cultures using Anansa® Fast .n. Easy Genomic DNA purification kit (Tebu-Bio Laboratories, Cedex, France) and the method described by Boom *et al*. (1990). The quantity of extracted DNA was estimated by electrophoresis in 1.5 % agarose stained with ethidium bromide through comparing the intensity of the genomic DNA bands with a standard DNA size marker (100-1000 bp, Smart ladder®, Eurogentec, Belgium). The primers used were G1 (5'-CTGAATCGCTGTATAAAAGT-3') G2 (5'-GGAAAACA AATGGTCGGAAG-3'). These primers are traditionally known to be diagnostic of leptospirosis and were described by Gravekamp *et al.* (1993). PCR amplification was carried out in a total volume of 25µL,

Amplification was processed in a thermocycler with an initial cycle of 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 51°C for 1 min and 72°C for 1 min, with a final

extension of 72°C for 7 min. The PCR product was analyzed by electrophoresis in 1.5% agarose gel, stained with ethidium bromide (0.5 mg/ml) and visualized by UV trans-illuminator

4.2.7 Renal histopathological changes in rats with naturally infected with leptospirosis

Samples of the kidney of the trapped rats were fixed in neutral-buffered formalin. Tissues were then dehydrated in graded levels of alcohol and embedded in paraffin wax, sectioned at 5µm and stained with Heamatoxylin and alcohol eosin Warthin starry silver stain were also used to stain some of the sections. The degree of the histopathological lesions in the kidneys sections were recorded as +++ = severe or marked, ++ = moderate + = mild and - = absent. The presence of the leptospiral organism observed by Warthin Starry silver stain and immunohistochemistry was recorded as severe (+++ : >60%), moderate (++: 40 to 60%) and mild (+ : <40%) according to the number and percentage of the tubules in 6 different (2 cortical, 2 corticomedullary junction and 2 medullary) microscopic fields. Rats were considered infected if leptospiral organisms were detected in the kidney sections at least by one of the 2 diagnostic techniques used in this part of the study.

4.2.8. Immunohistochemical Staining of leptospiral positive tissues

Serial 5-µm sections from each paraffin block were mounted on slides and allowed to dry overnight.

The sections were deparaffinized in 4 changes of xylene for 3 min each, rehydrated in a graded series of ethanol solutions (100%, 95%, 70%, 50%), and finally washed with deionized water.

The deparaffinized sections were then placed in a Coplin jar containing 0.01 M sodium citrate buffer (pH 6.0) and heated for 40-mins periods, followed by cooling to room temperature for 20 mins, to unmask the antigens.

Following 5 min of washing in 0.05 M Phosphate-buffered saline (PBS) solution (pH 7.6) containing 0.05% Tween 20 (TPBS). All steps were carried out at room temperature.

After a brief wash of TPBS, nonspecific binding was blocked by bathing in normal goat serum (1:10 dilution in PBS) for 10 min.

The tissues were then incubated with rabbit monoclonal (1:800 dilution in PBS) for 30 min.

After a brief wash with TPBS, the slides were incubated with biotinylated secondary antibody (goat anti-rabbit IgG) for 15 min.

After a last wash in TPBS, the slides were incubated with streptavidin–Alkaline phosphatase for 15 min. Slides were then rinsed with distilled water and incubated with fast red chromogen for 10 min. Slides were final rinse with distilled water to stop the staining process.

Sections were counterstained with haematoxylin for 3 min followed by a 5-min rinse in running tap water and were mounted with glycerol water-soluble mounting medium for microscopic evaluation. Negative controls were slides from Leptospira-infected tissues that were subjected to the same staining procedure as the others except that normal rabbit serum was used in place of the monoclonal antibody.

4.2.9 Effects of age/size on the leptospiral tubular colonization in rats

Out of the 105 rats in this study, the number of adults and juveniles were determined, with the number and prevalence of the leptospire in kidney sections in each group, using EMJH medium and Warthin Starry silver stain. The degree of the tubular colonization was determined at x 400 magnification under the Olympus microscope using; + = mild for 1 to 2 colonized tubules, ++ = moderate for 3 to 5 colonized tubules and +++ = marked for >6 colonized tubules per field.

4.2.10 Statistical analysis

Percentages of both positive and negative cases were determined for different levels of age, year, sex and breed.These data were summarised using proportions with 95% confidence intervals calculated using the Wilson method as described by Brown *et al*., (2001).

Data were also represented using bar-charts. Student t-test was used to determine the significance between infection and the observed microscopic lesions, performed by Analyse-it for Microsoft excel (Analytical software). P value of <0.05 was considered statistically significant.

4.3 RESULTS

4.3.1 Body and kidney appearance of the captured rats.

All the trapped rats were externally normal, except in few rats that showed scars on the legs and few areas of alopecia on their skin. No external parasites were observed on their bodies. They all revealed normal mucous membranes. All the kidneys examined in this study were normal with the characteristic chocolate colour, except in one rat in which there was moderate pallor of the cortical surface.

4.3.2 Bacteriology and characterization

Table 4.1 depicts the prevalence of leptospiral organisms in 105 kidneys of rats in the three cities using EMJH medium. A total of 105 rat kidneys were examined culturally for the presence of leptospiral organism. Out of the 105 kidney samples examined in this study, 12 (18.2%), 49 (46.7%) and 44 (41.9%) were from Lagos, Ibadan and Abeokuta respectively. Out of the 12 kidneys from Lagos, 8 kidneys (66.7%) were culturally positive and 4 (33.3%) were negative. Thirty-seven (75.5%) were positive and 12 (24.5%) were negative out of the kidneys from Ibadan and 27 kidneys (61.4%) were positive and 17 (38.6%) were negative from Abeokuta. In all the 105 kidney samples, 72 (68.6%) samples were culturally positive in the EMJH medium, while 33 (31.4%) kidneys were negative. Contaminations of some of the isolates were observed during the course of this study.

Out of the 105 captured rats, 74 (70.5%, 95%CI from 66 – 74%) and 31 (29.5%, 95%CI from 21 – 39%) were males and females respectively. Fifty-five kidney samples (55.4%, 95%CI from 45 – 65%) were culturally positive to leptospiral infection out of the 74 male rats captured compared with 19 (18.1%, 95%CI from 11 – 25%) negative male rats, while 17 (16.2%, 95%CI from 9 – 23%) were positive out of the 31 female rats examined, while 14 (13.3%, 95%CI from 7 – 19%) were negative.

Table 4.1. Showing the prevalence of leptospiral organisms in 105 kidneys of rats in the three cities using EMJH medium

			95%CI			95%CI			95%CI
Cities	No	%	Lower-Upper	lepto +ve	%	Lower – Upper	Lepto -ve	%	Lower - Upper
Lagos	12	11.4	05 – 17	08	66.7	40 – 94	4	33.3	06 – 60
Ibadan	49	46.7	37 - 57	37	75.5	64 – 88	12	24.5	37 – 61
Abeokuta	44	41.9	33 - 51	27	61.4	47 – 75	17	38.6	25 – 61

Lepto -ve= leptospire negative, Lepto +ve = leptospire positive.

Table 4. 2 Distribution of leptospiral infected captured rats according to sex. Values of 95%CI were also included

	Total No	(%)	95%CI Lower upper	Lepto +ve	(%)	95%CI Lower upper	Lepto -ve	(%)	95%CI Lower upper
Males	74	70.5	66 - 74	55	55.4	45 - 65	19	18.1	11 - 25
Females	31	29.5	21 - 39	17	16.2	9 - 23	14	13.3	7 - 19

Lepto -ve= leptospiral negative, Lepto +ve = leptospiral positive.

Table 4. 3 Distribution of leptospiral infected rats according to Age/size with the degree of tubular colonization in the affected rats, using culture isolation and Warthin Starry silver stain.

	Total no (n=105)	No +ve (n=72)	Prevalence (%) (68.6%)	Severity of the tubular colonization		
				+	++	+++
Juveniles	35	21	20.0	19	2	-
Adults	70	51	48.6	7	9	35

+ = mild, ++ = moderate, +++ = marked.

Out of the 105 rats examined, adult rats had the highest prevalence of leptospiral infection, in which 51 (48.6%) out of 70 adult rats were positive compared with the juveniles with 21 (20%) out of 35 that were positive to leptospiral infection. Using Warthin Starry silver stain the tubules of the adult rats showed more severe tubular colonization (Figs.4.3-4) than the juveniles (fig.4.13) with 35 kidney sections showing severe tubular colonization, compared with juveniles without any severe tubular colonization. Juveniles were more that showed slight tubular colonization (19 kidney sections) than the adults (7 kidney sections) that were slightly colonized. Nine and 2 kidney sections were moderately colonized in the adult and juvenile's kidney sections respectively.

Out of the 72 kidney samples positive to leptospiral organism by the EMJH medium, only 17 (23%) uncontaminated samples were used. Twenty-one serotypes of leptospiral organism were determined from 17 rat's kidney in this study. This shows that 4 rats out of the 17 rats harboured more than 1 serotype of leptospiral organism in their kidney. Seven (33%) out of the 21 isolates were *L. icterohaemorrhagiae* with the highest prevalence and agglutination titre of 1:1600. This was followed by *L. Pomona* with 5 (23.8%) rats and agglutination titres of 1:3200. *L. grippotyphosa* and *L. canicola* were 3 (14.3%) rats each, but with different agglutination titres (1:3200 and 1: 800 respectively). *L. Bratislava* was present in 1 rat (4.8%) with agglutination titres of 1: 1600.

Table 4. 4 Characterization and prevalence of leptospiral Isolates using monoclonal antibodies in microscopic agglutination test (MAT) with 17 positive kidney samples of wild rats

No	Serotypes	No. positive	Prevalence (%)	95%CI Lower - Upper	Agglutination titers
1	*L. Pomona*	5	23.8	6 - 42	1: 3200
2	*L. Grippotyphosa*	3	14.3	00 - 39	1: 3200
3	*L. Hardjo*	-	-	–	-
4	*L. Bratislava*	1	4.8	00 - 13	1: 1600
5	*L. Canicola*	3	14.3	00 - 39	1: 800
6	*L. Icterohaemorrhagiae*	7	33.0	13 - 46	1: 1600
7	*Undetermined serovars*	2	9.5	00 - 23	-

4.3.3 Detection of pathogenic leptospiral DNA in kidneys of infected wild rats using Polymerase chain reaction

Eleven kidney samples from the house and city rats were subjected to PCR analysis to determine the virulence gene of the leptospiral organisms using specific pathogenic primers (G1/G2). Out of the 11 kidney samples, only 9 (81.8%, 95%CI from 59 to 100%) was positive (lanes 3, 4, 5, 6, 7, 8, 9, 10 and 11) and showed amplification of the leptospiral gene which is between 285bp to 300bp. Two (18.2%, 95%CI from 15 to 21%) kidney samples (lanes 1 and 2), out of the 11 kidneys were negative and did not showed any amplified product from the affected house rat which were positive in the EMJH medium. The leptospiral amplicons or the amplified products were slight to moderate in intensity.

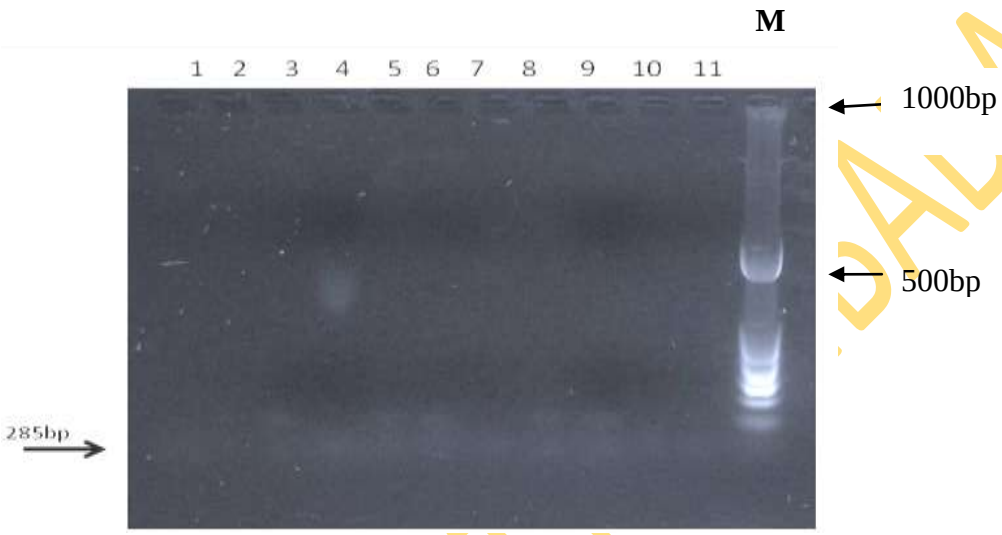

Fig.4.1 Photograph of the 1.5% agarose gel stained with ethidium bromide showing product specific PCR obtained with DNA extracted from kidney samples of rats naturally infected with leptospire species. Lane M= molecular maker 1kb DNA ladder, Lanes 1 to 7 positive kidney samples of rats.

Table 4. 5 Total numbers and histopathological changes in kidneys of leptospiral positive and negative rats in the three cities.

Lesions	Total (n=105)	%	95%CI Lower-upper	lepto+ve (n= 72)	%+ve	95%CI Lower - upper	lepto-ve (n=33)	%-ve	95%CI Lower-upper
Tubular degeneration and necrosis	91	86.7	81 – 93	71	67.6	59 – 77	20	19.1	11 - 27
Interstitial lymphoplasmacytic infiltration	76	72.4	63 – 81	66	62.9	54 – 72	10	9.5	04 – 16
Tubular hyaline casts	67	63.8	55 - 73	51	48.6	39 – 59	16	15.2	08 – 22
Glomerular casts	62	5.91	50 – 68	42	40.0	31 – 49	20	19.1	11 - 27
Tubular dilatation	62	59.1	50 – 68	48	45.7	36 – 56	14	13.3	07 – 19
Interstitial fibrosis	30	28.6	20 – 38	20	19.1	11 – 27	10	9.5	04 – 16
Interstitial oedema	14	13.3	07 - 19	12	11.4	05 – 17	02	02.0	00 – 05
Membranous glomerulonephritis	36	34.3	25 – 43	26	24.8	17 – 33	10	10.0	04 – 16
Tubular calcification	11	10.5	05 – 17	05	04.8	01 – 10	06	05.7	01 – 11
Pylonephritis	04	04.0	01 - 07	02	02.0	00 – 05	02	02.0	00 – 05
Membranoproliferative glomerulonephritis	02	02.0	00 – 03	01	01.0	00 – 03	01	01.0	00 – 03
Embolic glomerulonephritis	01	01.0	00 – 03	00	00.0	00 - 00	01	01.0	00 – 03

Lepto +ve = leptospiral positive, lepto-ve = leptospiral negative,

Table 4. 6 Semi-quantification of the degree of histopathological lesions in the kidneys of leptospira positive and negative rats in each of the three cities

	Lagos n = 12 (11.4%)		**Ibadan n= 49 (46.7%)**		**Abeokuta n=44 (41.9%)**	
Lesions	**Culture + 8 (66.7%)**	**Culture - 4 (33.3%)**	**Culture + 37 (75.5%)**	**Culture - 12 (24.5%)**	**Culture + 27 (61.4%)**	**Culture – 17 (38.6%)**
Tubular degeneration and necrosis	+++	++	+++	++	+++	++
Interstitial lymphoplasmacytic infiltration	++	-	++	-	++	+
Interstitial fibrosis	+	-	+	++	++	++
Tubular hyaline casts	++	+	++	++	++	++
Glomerular casts	+	-	+	++	+	++
Tubular dilatation	++	+	+	++	+	++
Interstitial oedema	+	-	-	-	+	+
Pylonephritis	+	-	-	++	+	+
Membranoproliferative glomerulonephritis	-	-	-	++	+	-
Membranous glomerulonephritis	++	+++	+	+++	+	++
Embolic glomerulonephritis	-	-	-	-	-	++
Tubular calcification	+	++	+	++	+	-

- = absent, + = mild, ++ = moderate, +++ = severe/marked. Culture+ = culture positive, culture - = culture negative

4.3.4 Histopathological changes in rats naturally infected with leptospirosis

Histopathological changes in the kidneys of rats with positive and negative leptospiral organism, using haematoxylin and eosin and Warthin Starry silver stains are depicted in Table 4.5 and Figs 4.2-3.

Microscopically, histological lesions were observed in both leptospiral positive and negative kidneys of rats. Tubular degeneration and necrosis was the most common lesion in the 105 rats examined in this study, being detected in 91 (86.7%, CI of 81- 93%) kidney of rats, in which 71 (67.6%, CI from 59-72%) were positive to leptospire infection compared with the remaining 20 (19.1% CI from 11- 27%) rats which were negative. Evidence of regeneration such as binucleation and cytomegaly were observed in tubular epithelial cells of some positive kidney sections.

Interstitial nephritis, being one of the lesions commonly associated with leptospiral infection, was present in 76 (72.4%, CI from 63 – 81%) rats, with varying degree of interstitial, perivascular, and periglomerular lymphoplasmacytic cellular infiltration. Out of the 76 rats that showed interstitial nephritis, 66 (62.9%, CI from 54 – 72%) were positive to the infection, while 10 (9.5%, CI from 04- 16%) rats were without inflammatory infiltrate, but showed slight to marked colonized tubules by leptospiral organism (Figs.4.4). The degree and distribution of the cellular infiltrate was mild to moderate with multifocal to coalescing, diffuse cellular infiltrate.

Membranous glomerulonephritis was observed in 36 rats (34.3%, CI from 25 – 43%), while 2 (2%, CI from 0-5%) rats showed membranoproliferative glomerulonephritis and 1 (1%, CI from 0-3%) with embolic glomerulonephritis. Multiple foci of tubular dystrophic calcification were observed in 11 kidney samples of rats (10.5%, CI from 5-17%), out of which 5 (4.8%, CI from 1-10%) rats were positive to the infection while 6 rats (5.7%, CI from 01 – 11%) were negative. Tubular hyaline cast was present in 67 (63.8%, CI from 55 –

73%) rat kidney sections, out of which 51(48.6%, CI from 39-59%) were positive to leptospiral infection while 16 (15.2%, CI from 8 – 22%) rat kidneys were negative. Glomerular casts and tubular dilatations were observed in 62 rats (59.1%, 95%CI from 50-68%), but 42 rats (40%, 95%CI from 31 – 49%) and 48 rats (45.7%, 95%CI from 36 – 56%) were positive to leptospiral infection, while 20 rats (19.1%, 95%CI from 11 – 27%) and 14 rats (13.3%, 95%CI from 7 – 19%) were negative respectively. Interstitial oedema was present in 14 rats (13.3%, 95%CI from 7 – 19%), out of which 12 rats (11.4%, 95%CI from 5 – 17%) were positive and 2 rats (2%, 95%CI from 0 – 5%) were negative to leptospiral infection. Pyelonephritis was also observed in 2% of the culture-positive rats and 2% of the culture negative rats.

Sixty-six (62.9%) out of the 105 rats examined in this study demonstrated leptospire in the tubular lumina of the renal cortex, medulla and the cortico-medullary junction of the kidney sections, using Warthin starry silver stain. Immunohistochemically, 6 (50%) kidney samples were positive out of the 12 sample tested. Four (33.3%) of the selected 12 kidney sections were positive to *Leptospira icterohaemorrhagiae* and 2 (16.7%) to *Leptospira canicola.*

There was significant difference in the renal histopathological changes of the leptospiral positive rats compared with those that were negative (28.67±7.51 and 9.33±2.05 respectively).They were also positively correlated (r = 0.87 with P value of 0.001).

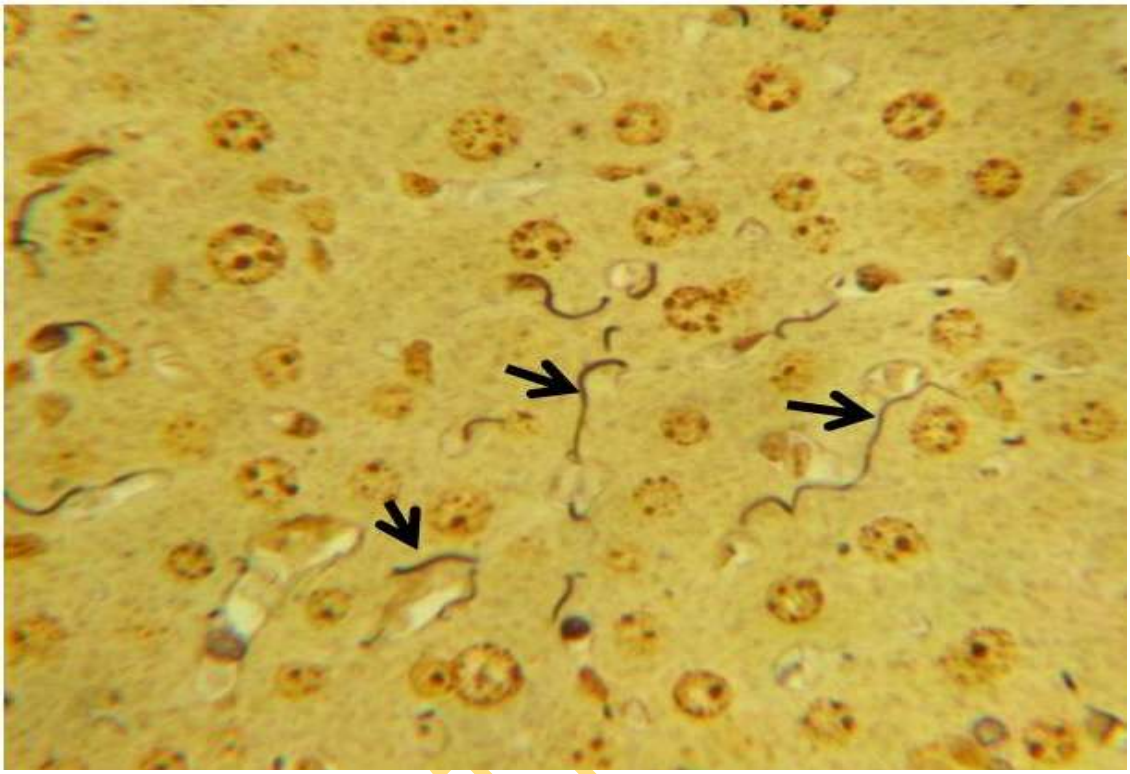

Fig.4.2. Photomicrograph of the liver of one of the naturally-infected wild rat showing leptospiral organism within the sinusoids (arrows). Warthin Starry silver stain. x 350.

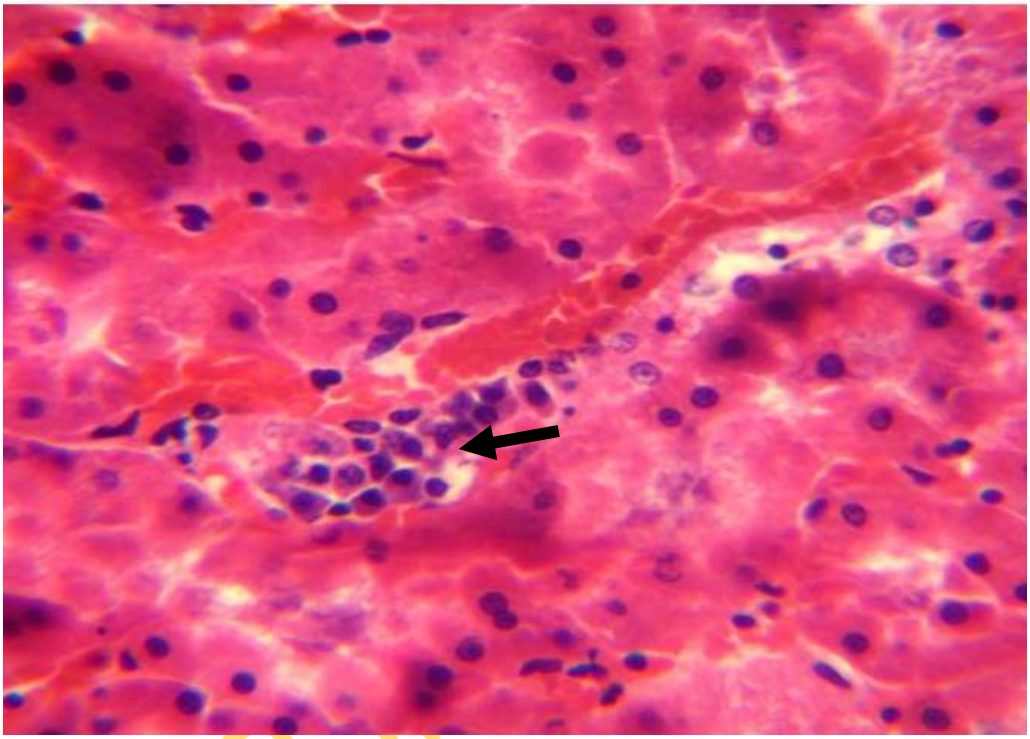

Fig.4.3. Photomicrograph of a kidney section showing moderate diffuse tubular degeneration and necrosis and focal area of interstitial lymphoplasmacytic cellular infiltration (arrow) in one of the wild rat positive with *Leptospira interrogans* serovar *canicola* infection. H & E. x 400,

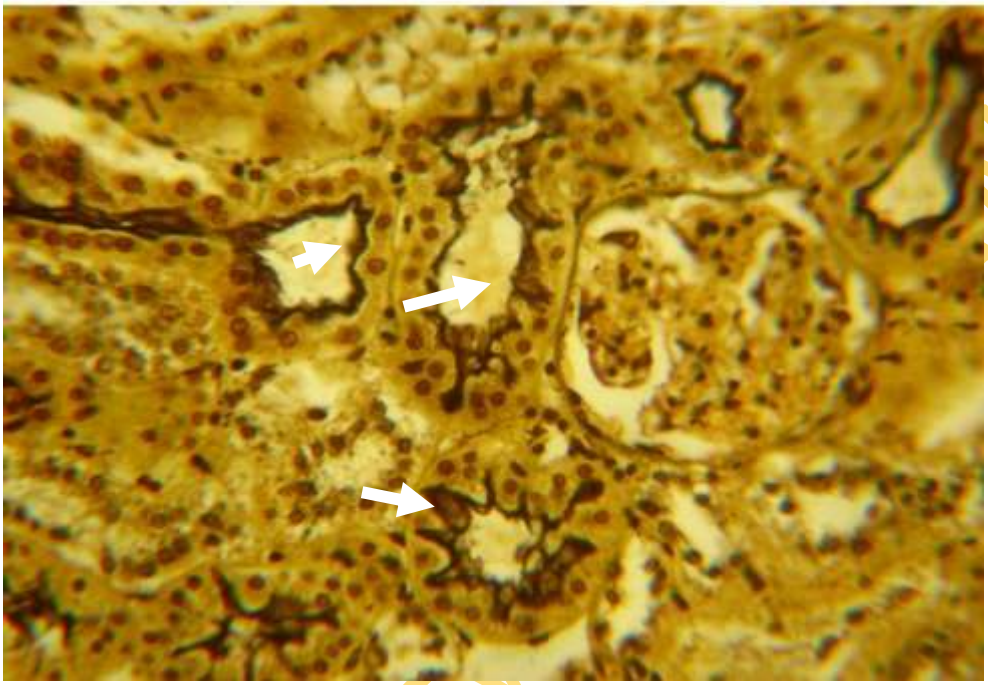

Fig.4.4 Photomicrograph of the leptospire infected kidney section ofwild rat with severe colonization of almost all the tubules (White arrows) and absence of interstitial mononuclear cells infiltration. Warthin Starry silver stain. x 300

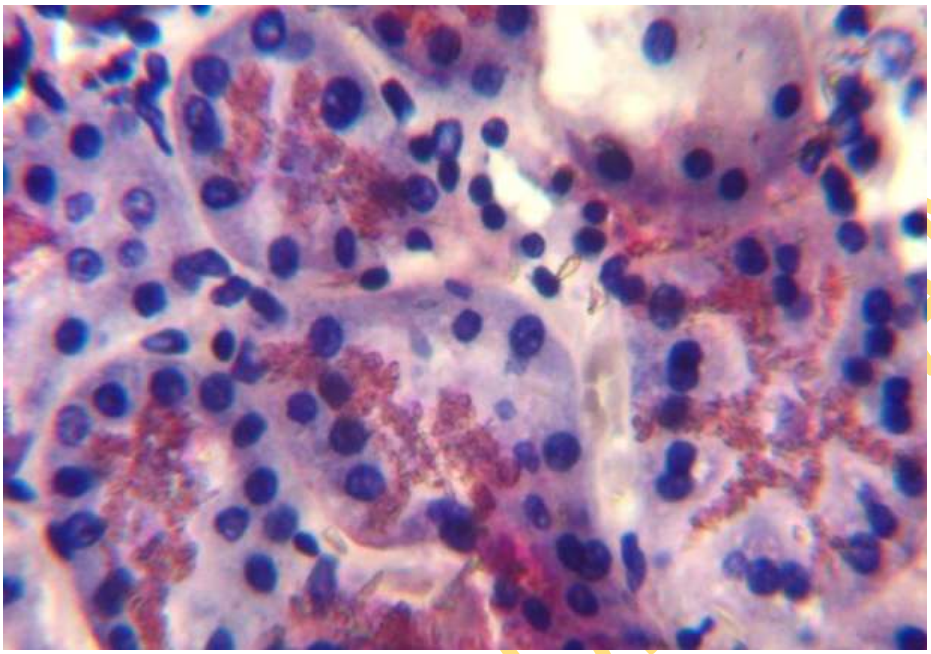

Fig.4.5 Photomicrograph of the kidney section of rat showing immunoreactivity of *Leptospira interrogans* serovar *icterohaemorrhagiae* antigen in the tubular lumen. Alkaline phosphatase-fast red counterstained with haematoxylin. x 400.

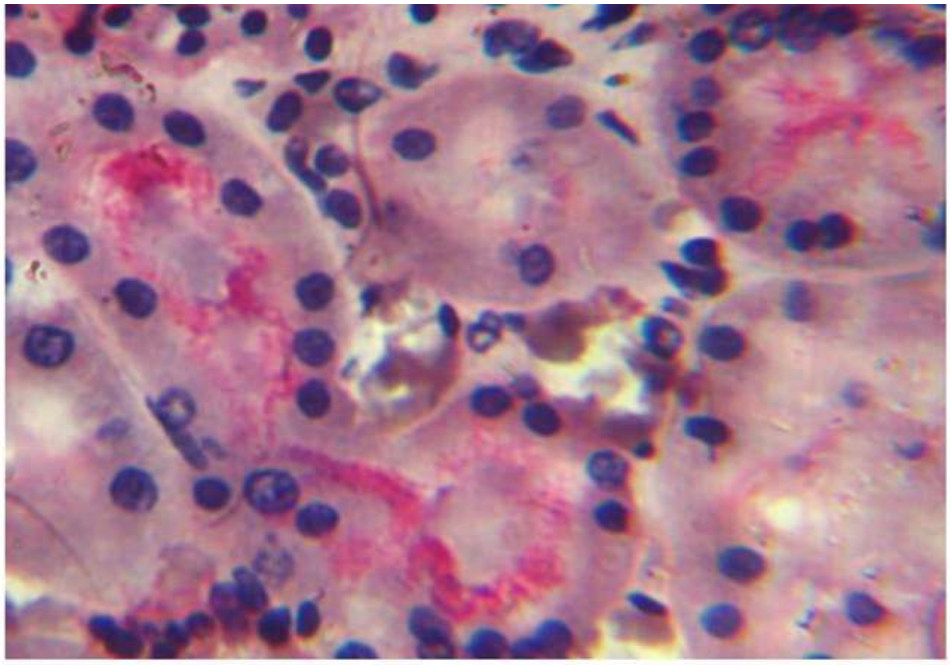

Fig. 4.6 Photomicrograph of the kidney section of rat showing immunoreactivity to *leptospira interrogan* serovar *canicola* antigen in both tubular lumina. Alkaline phosphatase-fast red counterstained with haematoxylin. x 450.

4.3.5 Regional and tubular localization with the degree of colonization of leptospiral organism within the renal parenchyma.

Out of the 72 kidney of rats positive for leptospirosis, 20 well colonized kidneys were selected and examined for the regional distribution and the degree of tubular leptospiral colonization (Table 4.7.), using slides stained with Warthin starry silver stain. The tubular locations include Bowman's space, proximal convoluted tubules, distal convoluted tubules and the collecting tubules while the regional locations were the cortex, cortico-medullary junctions and the medulla. Out of the 20 slides examined on the tubular distribution, only 1 (5%) rat's kidney demonstrated slight leptospiral organism in the Bowman's space mixed with the glomerular protein cast. Six kidneys (30%) of rat showed the presence of the organism within the proximal convoluted tubules. Eleven kidney sections (55%) showed mild to severe colonization of the organism within the distal convoluted tubules and 2 (10%) kidney sections demonstrated the presence of the organism in the collecting tubules. On the regional distribution, cortico-medullary junction demonstrated the highest area of leptospiral presence with 14 (70%) kidney sections and varying degree of tubular colonization. Four (20%) kidney sections showed more of the tubular colonization within the cortical area and 2 (10%) kidney sections in the medulla. Although, some of the kidney sections used in this study showed dual or triple tubular and regional localizations within the same kidney sections, but they were more concentrated on one location than the others.

Table 4. 7. Regional and tubular localization with the degree of colonization of leptospiral organism within the renal parenchyma.

Serial number	Locations within the renal parenchyma	No involved (20)	%	Degree of colonization		
				Mild (+)	Moderate (++)	Severe (+++)
	Tubular colonization					
1	Bowman's space (cortical area)	1	5	1	-	-
2	Proximal convoluted tubules (cortical area)	6	30	3	1	2
3	Distal convoluted tubules	11	55	2	2	7
4	Collecting ducts	2	10	1	1	-
	Regional colonization					
1	Cortex	4	20	1	1	2
2	Cortico-medullary junction	14	70	3	2	9
3	Medulla	2	10	1	1	-

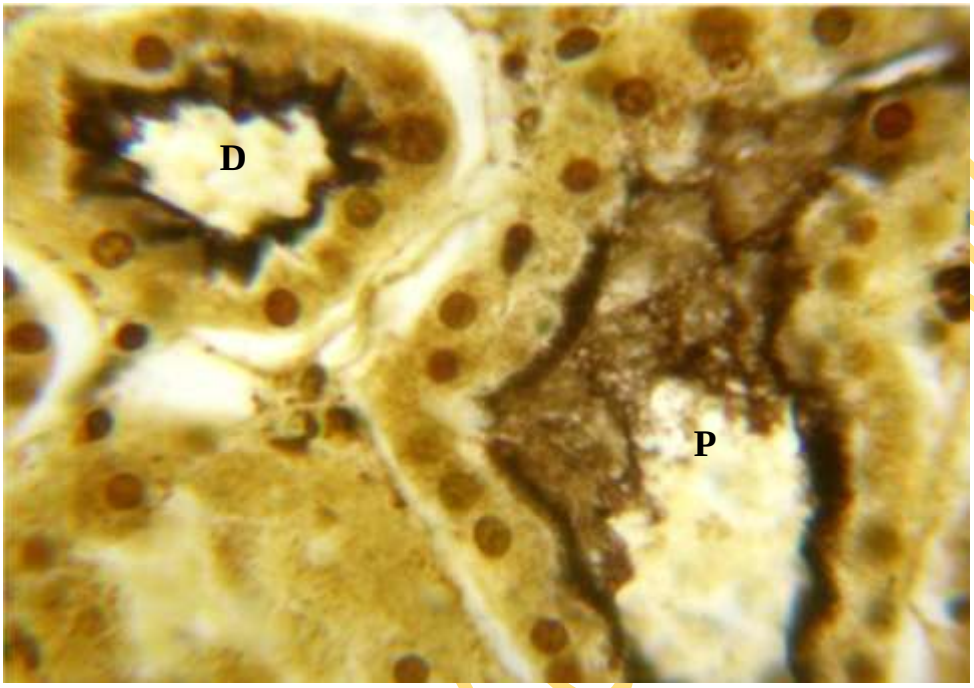

Fig.4.7 Photomicrograph of the leptospira infected kidney of rat with severe leptospiral colonization of distal (D) and proximal (P) convoluted tubules. Warthin Starry silver stain. x 450.

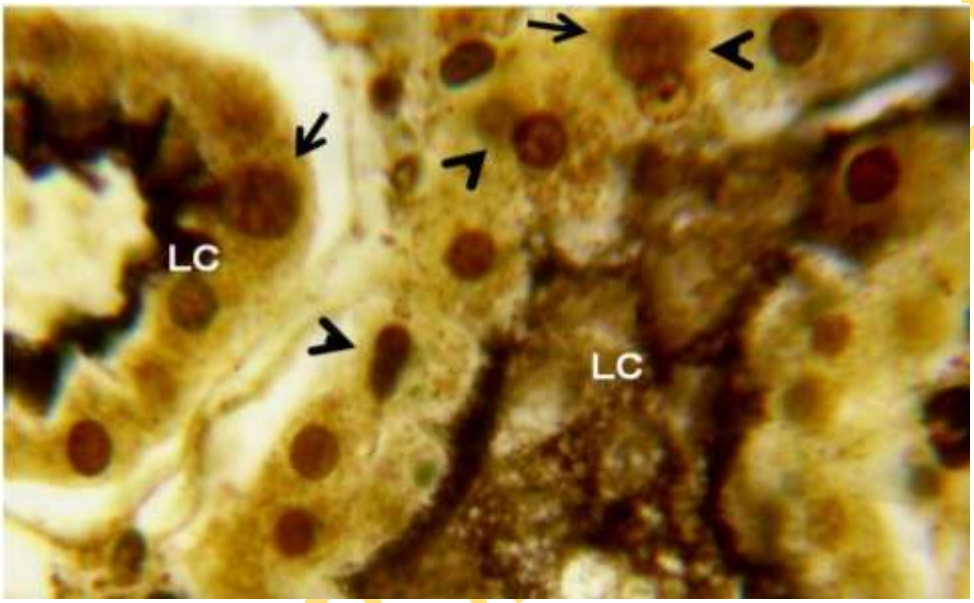

Fig.4.8 Photomicrograph of the leptospira infected kidney of rat with severe leptospiral colonization of distal and proximal convoluted tubules with regenerative changes such as cytomegaly (arrows), and binucleation (head arrows) Warthin Starry silver stain. x 650.

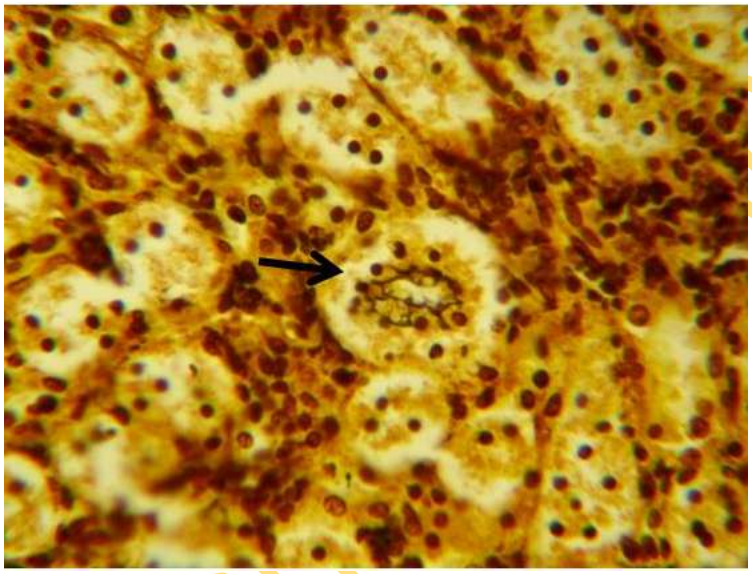

Fig.4.9 Photomicrograph of the kidney section of one juvenile wild rat showing severe diffuse tubular degeneration and necrosis with leptospire colonization of one collecting tubule (arrow), Warthin Starry silver stain. x 300

Table 4. 8 Comparative results of the five diagnostic techniques for the detection of Leptospiral organism in kidney and liver and the presence of microscopic lesions in infected house rats

Organs	EMJH	%	WSSS	%	IH	%	PCR	%	MAT	%
Kidney	72/105	68.6	66/105	62.9	6/12	50	9/11	81.8	15/17	88.2
Liver	ND	-	1/105	2.4	ND	-	ND	-	-	-

4.4 DISCUSSION

Out of the 105 rats captured in the 3 cities of the southwestern Nigeria, more than 68% were infected with pathogenic leptospiral spp. compared with other surveys (apart from the studies of Tucunduva de faira *et al.*, 2008) with carriage rates ranged from 4 to 59% when isolated in the EMJH medium (Thiermann, 1977; Pereira and Andrade, 1988; Taylor *et al.*, 1991; Webster *et al.*, 1995; Levett *et al.*, 1998; Vado-Solis *et al.*, 2002; Sharma *et al.*, 2003; Vanasco *et al.*, 2003; Priya *et al.*, 2007). This study detected a much higher proportion (>68%) of leptospiral carriage among captured city rats more than the study of Diallo and Dennis, (1982), whose 4.5% of the 252 field rats caught in Zaria, Nigeria, were positive to leptospiral infection.

Contamination has been one of the major drawbacks of culturing using EMJH medium as it reduces the sensitivity of culture isolation of leptospiral organism (Tucunduva de faira *et al.*, 2008). This is in agreement with this study and possibly more of the rat's kidney would have been positive if some of the culture were not contaminated.

Divergent views abound in the literature as to the duration of carrier state of leptospirosis in rats. This appears to be dependent on age and the serovar of leptospire involved (Thiermann, 1981). Moreso, the effect of age or size of rat and the leptospire serovar involved on the degree or the severity of tubular leptospiral colonization in the kidney of rats is unknown. In the works of Athanazio *et al.*, (2008), chronically infected rats were studied for up to 120 days and were all positive by culture, silver staining and immunofluorescence. Previous studies have also shown that rats infected with serogroup icterohaemorrhagiae strain maintained renal carrier state for longer period of 220 days, compared with serogroup gripptotyphosa strain which was only maintained for 40 days (Thiermann, 1981). In this study, it is possible that age and leptospiral serovar involved might have played a significant role in the degree and number of tubular colonization as adult male rats showed more of the

tubular colonization in term of severity and the number of tubules involved (more than 8 colonized tubules at field magnification of x 300, Figs.4.6-7) than the juveniles (1 or 2 colonized tubules at X 300, Fig.4.13) and more of the serovar icterohaemorrhagiae was isolated and characterized in this study. The reason for these observations might be difficult to verify in this study, but it is possible to speculate that as the animals advances in age, more of the tubules become colonized and those tubules colonized previously become more densely populated.

Renal histopathological changes associated with chronic leptospirosis have been documented in different carrier animals including rats, dogs, pigs and wild animals (Morrison and Wright, 1976; Baker *et al*., 1989, Hamir *et al*., 2001; Yener and Keles, 2001; Tucunduva de faira *et al*., 2007). In an experimental infection with rat, interstitial nephritis was only observed after one month. This was characterized by mild to severe multifocal interstitial nephritis. (Monahan *et al.,* 2008). Similar findings have been documented by Tucunduva de faira *et al*., (2007) in which 56% of culture positive kidney of wild trapped rats showed distinct foci of interstitial nephritis. However, in the same study, similar inflammatory infiltrate were present in 43% of culture negative wild rats. In this study, histopathological changes of interstitial nephritis were observed in 62.9% of the cultured positive kidney samples and 9.5% of the culture negative samples. This is consistent with those documented in the literature on leptospiral infected animals (Yener and Keles, 2001; Tucunduva de faira *et al*., 2007), but because of the nature of this study it would be difficult to attribute all the lesions in the infected kidney as consequence of leptospiral infection as suggested by Rossetti *et al*., (2004).

The mechanism underlying the constant battle between maintenance host immune response and leptospire that colonized renal tubules during chronic infection remain elusive and poorly understood (Monahan *et al.,* 2009).

Various reasons have been given in the literature for the absence of interstitial nephritis in some heavily colonized rat's kidneys (Babudieri, 1958). These includes (1) the small area of each kidney sample examined, (2) the absence of damage in the kidney of some carrier animals and (3) the recovery of animals in which interstitial nephritis has subsided and cleared. This study is partly in agreement with these assertions, since 6 (8.3%) out of the 72 positive kidney samples were without interstitial nephritis (Table 4.5). Moreover, recently colonized tubules, different leptospire antigenic profiles that facilitate evasion of immune response (Monahan *et al*., 2009), formation of biofilm (i.e.colonies of bacteria encapsulated within a protective matrix which enable bacterial growth in hostile environmental conditions), colonization and immune evasion in the renal tubules during chronic infection (Hall-stoodley *et al.,* 2004) and the presence of leptospiral endostatin-like (Len) protein which bind complement regulatory proteins or promotes deactivation of the complement activation system, thus preventing leptospiral destruction (Lambris, *et al.,* 2008) have been suggested as the major causes of lack of interstitial nephritis in some culture positive rats.

The most common histopathological change in this study was tubular degeneration and necrosis being detected in 67.6% of the culture positive kidneys and 19.1% of the culture negative rats. This is closely followed by interstitial nephritis, being detected in 62.9% in the culture positive rats and 9.5% in the culture negative rats. This is contrary to the report of Tucundava de faira *et al.,* (2007) who observed tubular epithelial hyaline droplets as the most common histopathological findings in 64% of the culture positive and 87% of the culture negative rats. They also stated that in a population of a high rate of leptospiral infection, the findings of interstitial nephritis and other lesions cannot be attributed to leptospiral infection alone, since the same lesion were observed in the culture negative rats. Thus, the presence of interstitial nephritis in culture negative kidney samples might suggests causal agents which might be related to noxius environmental factors such as biological, chemical, and nutritional

factors. The aetiology of membraneous glomerulonephritis in the literature has been associated with immune mediated conditions (Newman *et al*., 2007). In this study, membraneous glomerulonephritis was observed in 24.8% of the culture positive rats and 10% in the culture negative rats. This is in agreement with the findings of Tucundava de faira *et al*., (2007) who documented 33% in glomerular alterations in culture positive captured rats and 20% in culture negative rats.

Previous studies have demonstrated cytomegaly, binucleation and multinucleated giant cells in the kidneys of naturally infected cattle as evidences of tubular epithelial regeneration (Hadlow and Stoenner, 1955; Burdin, 1963; Amatredjo *et al.,* 1976; Yener and Keles, 2001). This study is in agreement with works of these authors since evidence of tubular regeneration such as cytomegaly and binucleation in the severely colonized tubules were observed. Although some workers have attributed this to a foreign body reaction against degenerated tubules or their content (Amatredjo *et al.*, 1976), but the recent identification of leptospiral antigens in these giant cells possibly confirm that the findings of these workers were consistent with true response to persistent colonization.

There is a general concensus in the literature on the localization of leptospiral organism in the renal proximal convoluted tubules of the carrier animals (Miller and Wilson, 1967; Sterling and Thiermann, 1981) but Seibold *et al.* (1961) and Rossetti *et al.,* (2004) were of contrary opinion on the localization of the organism in cattle and house mice respectively. Seibold *et al.* (1961) observed the presence of leptospiral organism in the tubules of cortico-medullary junction in cattle with extensive inflammatory reactions. Rossetti *et al*. (2004) found in their study the presence of leptospiral organism on the apical surface of the epithelial cells in the lumen of medulla tubules but without focal or generalized tubular nephrosis and the associated inflammatory infiltrate. This study is in agreement with the works of Seibold *et al*. (1961) in cattle, since 14 (70%) out of the 20 rats examined showed

varying degree of tubular colonization at the cortico-medullary junction. But extensive inflammatory response was not present, only mild to moderate foci of inflammatory response were observed. Seibold *et al.* (1961) hypothesized that leptospires migrated to the cortico-medullary junction because of the damaged inflicted by the organism to the cortical tubules. On the contrary, this work is not in agreement with this hypothesis, since no such cortical damage was observed in the kidney of the positive rats.

In this study, leptospires were observed at various segments of the nephron such as Bowman's space, proximal convoluted tubules and distal convoluted tubules and at the regional level of cortical, cortico-medullary junction and the medulla. The presence of the organism in the Bowman's space and the liver in 2 (%) of the rat might possibly suggest leptospiremia (i.e. the acute phase of leptospirosis) in these rat. The distal convoluted tubules and cortico-medullary junction had the highest levels of leptospiral colonization being 11 (55%) and 14 (70%) respectively. The reason for the highest level of colonization in this region (distal convoluted tubules) is not known, but it is possible to speculate that (1) low hydrostatic pressure of glomerular filtrate in these areas might have cause leptospiral organism to adhere and proliferate in this region, (2) the presence of the necessary or required nutrients for the growth of the organism after the urinary filtrate might have passed through various stages of absorption and excretion within the renal tubules as suggested by Sterling and Thiermann (1981) and (3) possibly, the leptospire localization within the renal parenchyma is species specific i.e. different serovars of leptospires have a specific location for proliferation and growth within the renal parenchyma (Rossetti *et al.,* 2004). All these assertions might need to be verified in the future investigations.

Moreover, the persistent colonization of leptospire organism in the tubular lumina of the kidney might possibly be due to other factors apart from absence of complement within the tubules as suggested by Monahan *et al*., (2009). These include: 1) the possibility of existence

of cordial host-pathogen relationship between tubular epithelial cells and the colonized leptospiral organism. This can be further explain in two ways, (1) either by the fact that leptospire organism in the tubular lumina seemed not to cause much damage to the attached tubular epithelial cells in chronic infection as it is observed in this study (figs.4.6-7) or (2) constant process of regeneration of the degenerated tubular epithelial cells, which compensated for the dying or lost epithelium, evidenced by the cytomegaly and binucleation of the tubular epithelia cells (fig.4.12) observed in this study. This assumption might possibly be one of the mechanism by which leptospire organism survive in the tubular lumina. Further studies might be necessary to elucidate the effects of or the relationship between leptospiral organisms and the attached tubular epithelial cells in carrier animals.

Secondly, the absence of phagocytes in the tubular lumina might have also contributed to the mechanism of immune evasion of leptospire organism (even in the presence of complements as suggested by Monahan *et al*., 2007), since phagocytes were actively involved in the clearance of the organism in other organs such as the liver, lung and spleen (Monahan *et al*., 2009).

Thirdly, there is also the possibility that leptospire organism within the tubular lumina might have had a change or an expression of different antigenic profiles (antigenic protein downregulaton), different from the one the host immune system elicited immunoglobulin against, and consequently facilitates evasion of an immune response and thus enabling persistence leptospiruria as suggested by Monahan *et al*., (2009). Sequencing of the DNA of infected leptospiral organism and leptospire in the colonized tubules will elucidate this assertion.

Sterlling and Thierman (1981) have shown that rats excrete high level of leptospires in their urine (10^7 organisms per ml) for as long as 9 months after experimental infection. In the present study, although the quantity of leptospire organism in the urine of these rats were not

determined, but the severity and the numbers of renal tubules colonized by leptospiral organism suggest high concentration of the organism might have been excreted in the urine of these rats.

The applicability of PCR methodology in the diagnosis of leptospirosis has been emphasized in the literature (Vitale *et al*., 2007; Priya *et al.*, 2007). In the report of Rajeshwari *et al*., (2011), only 17.65% samples were found positive for the presence of leptospiral antigen by PCR in the urine of rats. Vitale *et al.,* (2007) discovered in Italy, 35% of the urban rodent's kidneys were positive for pathogenic leptospire using PCR. In this study, 9 (81.8%) out of the 11 leptospiral infected kidney gave an amplified DNA product of 285bp, although with variations and low intensity of the fragments. The remaining 2 samples failed to amplify any DNA products. This might probably be due to either intrinsic inihibitory factors during the process of the PCR or to the lower dectection limit of the G1/G2 primers under laboratory conditions as stated by Tucunvada de faira *et al.*, (2008).

Studies on the use of monoclonal antibodies in serotyping of leptospire organism to the serovar level are few in the literature. It has been adjudged to be one of the best methods to identify leptospiral isolates with relative rapidity (Shinozaki *et al.*, 1992).Suepaul *et al.,* (2010), found leptospira Copenhageni as the most prevalent serotyped in 37 (68.5%) out of the 54 rodents examined, using monoclonal antibodies in Trinidad. In this study, *Leptospire icterohaemorrhagiae* (7/17, 33%) and *Pomona* (5/17, 23.8%) were the most prevalent serovars out of the 17 leptospiral isolates examined using monoclonal antibodies, although 2 were undertermined.

CHAPTER FIVE

5.0 ISOLATION AND CHARACTERIZATION OF *LEPTOSPIRE SPP.* AND ASSOCIATED RENAL PATHOLOGY IN CATTLE FROM ABATTOIRS IN TWO SOUTHWEST STATES OF NIGERIA

5.1 INTRODUCTION

Studies on the prevalence of bovine leptospirosis in African continent are few. This has been attributed to the difficulties in recognizing the clinical manifestations and diagnosis of the condition in cattle; hence the diasease has not been well investigated (Mgode *et al*., 2006). Currently, 20 leptospiral serovars have been described in Africa. Eleven of these serovars belong to species *leptospire kirschneri* with eight being found in the Democratic Republic of Congo (Zaire), two in Kenya and one in Ghana (Faine *et al.*, 1999; Mgode *et al*., 2006). Mgode *et al.*, (2006) suggested that *L. kirschneri* might be the prevalent leptospire species, especially in the central and eastern Africa.

The clinical signs of bovine leptospirosis vary with the serovar: infections with serovar *hardjo*, for example, are not usually associated with hemolytic anemia. In adult cattle, the early symptoms such as fever and depression are often transient and milder, and may go unnoticed (Corney, 2004). The most prominent signs of infection are abortions, decreased fertility or decreased milk yield. Some serovars cause late term abortions, stillbirths and increased neonatal mortality (Alonso-Andicoberry *et al.*, 2001). The placenta is retained in up to 20% of the cows that abort, and infertility may be a sequela. Some serovars can cause sudden agalactia or decreased milk production (Alonso-Andicoberry *et al.*, 2001). The milk may be thick, yellow, and blood-tinged but there is typically little evidence of mammary inflammation (Mahmoud *et al.*, 2008). The appearance of the milk usually improves in 4 to 5 days, and milk production returns to normal after 10 to 21 days (Mahmoud *et al.*, 2008). Jaundice may be seen in severely affected animals.

Cattle are considered maintenance hosts of the serovar Hardjo (Moreira *et al.*, 2004). Serovar Hardjo has two distinct genotypes: Hardjobovis and Hardjoprajitno. The genotype Hardjobovis belongs to the species *Leptospira borgpetersenii,* while the genotype Hardjoprajitno belongs to the species *L. interrogans*. Both are important causes of reproductive disorders in cattle herds worldwide and have different clinical manifestations (Faine, 1999).

The accurate diagnosis of leptospiral infection in cattle is dependent on isolation and typing of the prevalent serovar (Mineiro *et al.*, 2011). However, only serological surveys have been conducted with few studies on cultural isolation in Nigeria. In most of the studies, MAT was used to detect antibodies against leptospires. However, this test has some limitations since it does not identify the specific genotype. Documented information on the role of cattle in the epidemiology of leptospirosis in Nigeria is scanty. However, there have been reported cases of leptospirosis in both the Southwestern and Northern part of Nigeria in rodents, sheep, goat and cattle (Diallo and Dennis, 1982; Agunloye, 2002). Agunloye, (2002) surveyed for leptospirosis in cattle, sheep and goat in the southwestern part of Nigeria, and a high prevalence was reported in these species of animal. Prior to this time, Agunloye, *et al.*, (1997) reported outbreak of leptospirosis in a goat herd in Ibadan.

Bacteriological survey of leptospirosis in Zaria, Nigeria (Diallo and Dennis, 1982) indicated that five isolates were recovered from 74 bovine kidneys examined, in which one was serovar pyrogenes and four unidentified. A new strain belonging to serogroup pyrogenes was recovered from 5 of 6 isolates collected from kidneys of cattle slaughtered at an abattoir in Nigeria (Ezeh *et al.*, 1989). Ezeh *et al.*, (1990) also isolated a new pathogenic serovar, called serovar *Nigeria* in the kidneys of cattle. In the serological survey of bovine leptospirosis carried out in Plateau State, Nigeria by Ezeh *et al.*, (1990), showed that out of the 1537 samples, 222 (14.4%) were positive to leptospirosis. The prevalence rates of antibodies to

individual serovars include: hardjo (35.6 %), pomona (11.7 %), pyrogenes (11.7 %), canicola (9.5 %), grippotyphosa (7.7 %), bratislava (5.9 %), icterohaemorrhagiae (5.9 %), ballum (4.5%), autumnalis (3.6 %), bataviae (2.3 %) and tarassovi (1.8 %).

In humans, serovar hardjoprajitno was isolated from the urine of a butcher serologically positive for leptospirosis in Nigeria which probably must have been acquired from cattle (Ezeh *et al*, 1991).

At present, little is known about the epidemiology of the infection and the possibility of both human and animal infection through cattle. In the studies of Okewole and Ayoola (2009) in dogs, they discovered that 42 (80.8%) out of the 52 dogs were on home-made foods dominated by abattoir offals. And they suggested the possibility of other animals especially dogs beign infected through this abattoir-offals home-made foods. Moreover, Barr, (2002) affirmed that kidneys of carrier animals and meat from animals slaughtered during leptospiremia and occasionally from diseased or convalescent cattle play an important role in the epidemiology of leptospirosis.

In the literature, different molecular methods have been employed for the specific detection of pathogenic leptospiral organism. These include in situ hybridization (Terpstra *et al.*, 1987), DNA–DNA hybridization (Terpstra *et al.*, 1986), and DNA probes (Lefebvre, 1987), which have been used mainly for detection of leptospires in urine samples from animals infected experimentally with serovar Hardjobovis. The polymerase chain reaction (PCR) also has been used to detect Leptospira spp. in urine samples from cattle experimentally infected with serovar Hardjobovis (Van Eys *et al.*, 1989; Gerritsen *et al.*, 1991; Wagenaar *et al.*, 1994, 2000; Taylor *et al.*, 1997; Alt *et al.*, 2001). PCR was also used to detect leptospire spp. in the urine of naturally infected cattle using genus-specific primers by Talpada *et al.*, (2003). Bomfim *et al.*, (2007) also used nested polymerase chain reaction (PCR) using primers from

the LipL32 sequence of Leptospira spp. to detect shedding of pathogenic leptospires in urine from naturally infected cattle.

In Nigeria, there are no empherical studies on the isolation and characterization of leptospiral infection with the associated renal pathology from naturally infected cattle, as the majority of data is limited to serology. Hence, this present work is designed to determine the prevalence and characterize leptospire *interrogans* in cattle, by isolation in EMJH broth and microscopic agglutination test (MAT) using monoclonal antibodies, being considered the best method in arriving at a definitive diagnosis of leptospirosis over the microscopic sero-agglutionation test (Santa Rosa, 1970; Faine *et al.,* 1999). Moreover, different methodologies of identification of the organism such as silver stain impregnation, polymerase chain reaction (PCR) and immunohistochemical staining would also be used to detect the presence of the organism in the kidney tissues. Renal pathological changes associated with leptospirosis in cattle would also be evaluated.

5.2 MATERIALS AND METHODS

5.2.1 Study location and data source

This study was carried out in Ibadan and Abeokuta the state capitals of Oyo and Ogun States respectively, in the south western Nigeria. The studied cattle were brought to these two states from different Northern parts of the country and from Niger Republic. The animals were slaughtered in the central metropolitan abattoirs in Ibadan (Oyo-State) and Abeokuta (Ogun-State) where more than 500 and 200 heads of cattle respectively, are slaughtered daily.

5.2.2 Sample Collection

One-hundred and eight kidney samples of cattle without known history, sacrificed at slaughter were selected for the present study. The study was carried between febuary to September 2009. The health status or on-going clinical conditions of the animals were not known, and animal were randomly selected. About 10-15g of kidney portions from each studied cattle were either purchased or given freely by the butchers. The kidneys were placed in individual sterile polythene bags, which were put into ice-pack and taken to the Department of Veterinary Pathology, University of Agriculture, Abeokuta within 3 – 4 hours for subsequent bacteriological and pathological analyses. A new set of sterile instruments were used for the sampling of each kidney.

5.2.3 Cultural isolation of leptospire organism from the kidney of naturally-infected cattle

Kidney samples were acquired using sterile scalpel blade, rat toothed forceps, Petri-dish and phosphate buffered saline (PBS). The isolation of le*ptospire* was performed by maceration of about 0.2-0.3g of kidney sample in 2 to 3ml of PBS with rat toothed forceps. This was allowed to stay for 10-15mins to allow the organism to move out of the tubules. Direct inoculation of one to two drops of the macerate into the 5 mL of Ellinghausen-McCullough-

Johnson-Harris broth medium (EMJH) (Difco®-USA) with the addition of 10 % of Rabbit serum and 5-fluorouracil (400 mg/L; Sigma®-USA and chloranphenicol (5 mg/L; Sigma®-USA), nalidixic acid (50 mg/L; Inlab®-BR), and neomycin (10 mg/L; Sigma®-USA) was performed.

The inoculated EMJH medium tubes were incubated at room temperature (28^0C – 30^0C) in the dark and examined under dark field illumination with intervals of 10 days to check for the growth of leptospires for at least 5 months.

When growth was observed, successive transfer was made in EMJH medium until the growth was sufficiently abundant. The number of bacteria was manually counted according to standard method recommended by WHO (Fanie, 1999).

5.2.4 Characterization of the cultured leptospiral isolates from kidney of cattle using monoclonal antibodies

Thirty-one pure cultures isolated from kidneys of cattle were characterized using leptospire monoclonal antibodies (Pomona, Bratislava, Hardjo, Grippotyphosa, Icterohaemorrhagiae and Canicola) kindly offered by the Royal Tropical Institute, Amsterdam. The microscopic agglutination test using monoclonal antibodies in this study was according to the method of Obregón *et al.*, (2007). Briefly, 50 µL of PBS was placed in each well from 2 – 6 of microtitre plate. Already prepared 50 µL monoclonal antibody at the dilution rate of 1:100 in PBS was placed in wells 1 and 2. Serial dilution of antibodies from well 2 to 6 were made in phosphate buffered saline (PBS) pH 7.2 in the microtitre plates. Then 50 µL aliquots of 7-10 days old culture of live leptospires in EMJH medium was added to each well. After 2-4 hrs of incubation at 37 ºC, the agglutination titre was determined by dark field microscopy.

5.2.5 DNA Extraction

DNA was extracted from the pure cultures of leptospiral organism isolated from kidneys of naturally infected cattle. Twenty-one isolates were used. Genomic DNA was extracted from the cultures isolates by the method described by Boom *et al.* (1990) using a commercial available DNA extraction kit (Quick-gDNA^{TM} Microprep, Zymo research, USA).

5.2.5.1 Primers

The primers used were G1 (5'-CTGAATCGCTGTATAAAAGT-3') and G2 (5'-GGAAAACA AATGGTCGGAAG-3'). These primers are traditionally known to be diagnostic of leptospirosis and were described by Gravekamp *et al.* (1993). PCR amplification was carried out in a total volume of 25µL,

Amplification was processed in a thermocycler with an initial cycle of 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 51°C for 1 min and 72°C for 1 min, with a final extension of 72°C for 7 min. The PCR product was analyzed by electrophoresis in 1.5% agarose gel, stained with ethidium bromide (0.5 mg/ml) and visualized by UV trans-illuminator

5.2.6 Pathology of kidney of cattle with natural leptospirosis

The remaining kidney sections were fixed in 10% buffered formalin after gross morphological observations. The tissue samples were processed routinely using haematoxylin and eosins stains and Silver Staining according to Young's modification of Warthin and Starry (1920) was used in identification of the Leptospires organism in the tubular lumina of the cattles. The degree of the histopathological lesions in the kidneys sections were recorded as +++ = severe or marked, ++ = moderate + = mild and - = absent. The presence of the leptospiral organism observed by Warthin Starry silver stain and immunohistochemistry was

recorded as severe (+++ : >60%), moderate (++: 40 to 60%) and mild (+ : <40%) according to the number and percentage of the tubules in 6 different (3 cortical and 3 medullary) microscopic fields. Cattle were considered infected if leptospiral organisms were detected in the kidney sections at least by one of the diagnostic techniques used in this study.

5.2.7 Immunohistochemistry

Serial 5-µm sections from each paraffin block were mounted on electrically charged slides and allowed to dry overnight. The sections were deparaffinized in 4 changes of xylene for 3 min each, rehydrated in a graded series of ethanol solutions (100%, 95%, 70%, 50%), and finally washed with deionized water. The deparaffinized sections were then placed in a Coplin jar containing 0.01 M sodium citrate buffer (pH 6.0) and heated for 40-mins periods, followed by cooling to room temperature for 20 mins, to unmask the antigens.

Following 5 min of washing in 0.05 M Phosphate-buffered saline (PBS) solution (pH 7.6) containing 0.05% Tween 20 (TPBS). All steps were done at room temperature.

The sections were first incubated in 3% hydrogen peroxide for 15 min to quench endogenous peroxidase. After a brief wash of TPBS, nonspecific binding was blocked by bathing in normal goat serum (1:10 dilution in PBS) for 10 min. The tissues were then incubated with rabbit monoclonal (1:800 dilution in PBS) for 30 min. After a brief wash with TPBS, the slides were incubated with biotinylated secondary antibody (goat anti-rabbit IgG) for 15 min.

After a last wash in TPBS, the slides were incubated with streptavidin– biotin–horseradish peroxidase for 15 min.

Slides were then rinsed with distilled water and incubated with 3-amino- 9-ethylcarbazole-peroxidase chromogen for 10 min. Slides were final rinse with distilled water to stop the staining process. Sections were counterstained with Mayer haematoxylin for 3 min followed

by a 5-min rinse in running tap water and were mounted with glycerol water-soluble mounting medium for microscopic evaluation. Negative controls were slides from Leptospira-infected tissues that were subjected to the same staining procedure as the others except that normal rabbit serum was used in place of the monoclonal antibody.

5.2.8 Statistical analysis

Percentages of both positive and negative cases were determined for different levels of age, sex and breed.These data were summarised using proportions with 95% confidence intervals calculated using the Wilson method as described by Brown *et al*., 2001.

Data were also represented using bar-charts. Student t-test was used to determine the significance between infection and the observed microscopic lesions, performed by Analyse-it for Microsoft excel (Analytical software). P value of <0.05 was considered statistically significant.

5.3 RESULTS

5.3.1 Prevalence of bovine leptospirosis according to location, sex and breed

Twenty-one (19.4%, 95%CI from12 – 26%) and 87 (80.6%, 74 – 88%) kidney samples were collected from the cities of Ibadan and Abeokuta abattiors respectively. Out of the 21 samples collected in Ibadan, 20 (95.2%, 95%CI from 86 – 100%) samples were leptospiral positive, while 1 (4.8%, 95%CI from 0 – 14%) sample was negative. Out of the 87 kidney samples collected at Abeokuta abattoir, 69 (79.3%, 95%CI from 70 – 88%) samples were positive to leptospiral infection, while 18 (20.7%, 95%CI from 12 – 30%) samples were negative.

Out of the 108 kidney samples randomly collected from the 2 abattoirs, female kidney samples were more than the male samples. Ninety-eight (90.7%, 95%CI from 86 – 96%) kidney samples were female out of the 108 samples collected in this study, while 10 (9.3%, 95%CI from 4 -14%) kidney samples were from male cattle. Out of the 98 female kidney samples, 79 (73.1%, 95%CI from 71 – 87%) kidney samples were culturally positive to leptospiral infection, while 19 (17.6%, 95%CI from 11- 27%) kidney samples were negative. All the 10 males (9.3%, 95%CI from 4 – 14%) kidney samples were culturally positive to leptospiral organisms.

Four different breeds of cattle were represented in the 108 samples collected from the 2 abattoirs. These include 76 (70.4%, 95%CI from 61 – 79%) White Fulani cattle, 9 (8.3%, 3 – 13%) Red Bororo, 4 (3.7%, 0 – 8%) Kuri cattle and 19 (17.6%, 11 – 25%) Muturu cattle. Out of the 76 White Fulani cattle, leptospiral organisms were isolated from 61 kidneys (56.5%, 48 – 66%), while the remaining 15 (13.9%, 95%CI from 7 – 14%) were negative. Leptospiral organism were also isolated from all the 9 (8.3%, 95%CI from 0 – 13%) kidney samples from the Red Bororo cattle, while non of the 4 (3.7%) kidney samples from Kuri breed of cattle were positive. All the 19 (17.6%, 95%CI from11 – 27%) kidney samples from the Muturu breed of cattle were positive to leptospiral infection.

5.3.2 Bacteriological studies

5.3.2.1 Cultural Isolation of leptospiral organism using EMJH medium.

Leptospire was successfully cultured from kidney samples of cattle from 24 hrs to 8 weeks of incubation which scored positively by dark-field microscopy with 10 cells motility per field at 24hrs early growth phase to as much as 5,000 cells with less motility per field at 5 weeks. Culturally, leptospires were isolated from 89 (82.4%) out of 108 kidneys from the naturally infected cattle. This consist of 20 (95.2%) out of 21 kidneys and 69 (79.3%) out of 87 kidneys collected from Ibadan and Abeokuta respectively (see table below). Out of the 89 positive culture kidney samples, 31 were uncontaminated and the remaining 58 samples were contaminated. The 31 isolates from the uncontaminated samples were used for the microscopic agglutination test. The remaining 19 samples were regarded as negative after 5 months of incubation.

5.3.2.2 Pathogenicity of the leptospiral isolates

Out of the eight guinea pigs used in this study to test for the pathogenecity of some of the isolates with two as the control, only one survived until day seven. At postmortem, they showed adhesion of the intestines with acute enteritis characterized by petechial hemorrhages. However, they reacted differently to the inoculate with seven of the guinea pigs coming down within 12-24 hours of inoculation with one surviving the infection beyond the proposed 4 days of euthanasia until day seven post infection but became dull and eventually died showing the previous ones.

5.3.2.3 Microscopic Agglutination Test using monoclonal leptospiral antibodies.

Table 5.1 below represents agglutination profiles of the 31 leptospiral isolates and their agglutination titres obtained from abattiors cattle.

Out of the 89 positive kidney samples in EMJH medium, 31 uncontaminated, 7-10 days old leptospiral isolates were chosen for the microscopic agglutination test using six panels of rabbit monoclonal antibodies. Out of the 31 isolates, 9 leptospiral isolates were *L. hardjo* with the highest prevalence (29%; 95%CI from 13% – 45%) and with the lowest agglutination titre (1:400). This is closely followed by *L. Bratislava*, with 7 isolates (22.6 %; 95% CI from 8% – 38%) and agglutination titre of 1:1600. *Leptospira grippotyphosa* and *L. icterohaemorrhagiae* were 3 (9.7%, 95%CI from 0.0 – 21%) isolates each, but with different agglutination titres (1:3200 and 1: 1600 respectively). *L. Canicola* had 2 isolates (6.5%, 95%CI from 0.0 – 16%) with agglutination titres of 1: 800. *L. Pomona* had 1 (3.2%, 95%CI from 0.0 – 09%), which was the least number of isolate with agglutination titres of 1:3200.

Table 5.1 Characterization and prevalence of leptospiral Isolates using microscopic agglutination test (MAT) in positive 31 kidneys samples of cattle.

No	Serotypes	No. positive	Prevalence (%)	95%CI Lower - Upper	Agglutination Titers
1	*L. Grippotyphosa*	3	9.70	0.0 – 21	1: 3200
2	*L. Hardjo*	9	29.0	13 – 45	1: 400
3	*L. Icterohaemorrhagiae*	3	9.70	0.0 – 21	1: 1600
4	*L. Bratislava*	7	22.6	08 – 38	1: 1600
5	*L. Canicola*	2	6.50	0.0 – 16	1: 800
6	*L. Pomona*	1	3.20	0.0 – 09	1: 3200
7	*Undetermined serovars*	6	19.4	05 – 33	-

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5.3.3 Detection of leptospiral DNA in the kidneys of infected cattle using PCR.

The amplifications of the leptospire DNA extracted from the kidney of cattle are depicted in Figs. 5.1-2. DNA was extracted from the culture leptospires from the kidneys of cattle naturally infected with leptospire spp. A total of 21 isolates were subjected to PCR analysis for the detection of 285bp amplicons. All the 21 isolates have been shown to be positive to pathogenic leptospire spp in the EMJH medium. Both forward (G1) and reverse (G2) primers described by Gravekamp *et al.*, (1993) for pathogenic leptospires were used. The PCR products amplified 285bp on 1.5% agarose gel. Out of the 21 isolates, only 13 (61.9%) were positive for PCR, while the remaining 8 isolates were negative. The PCR products amplified from the cultured leptospires have similar electrophoretic bands profiles, although they displayed variations in the intensity of the fragments

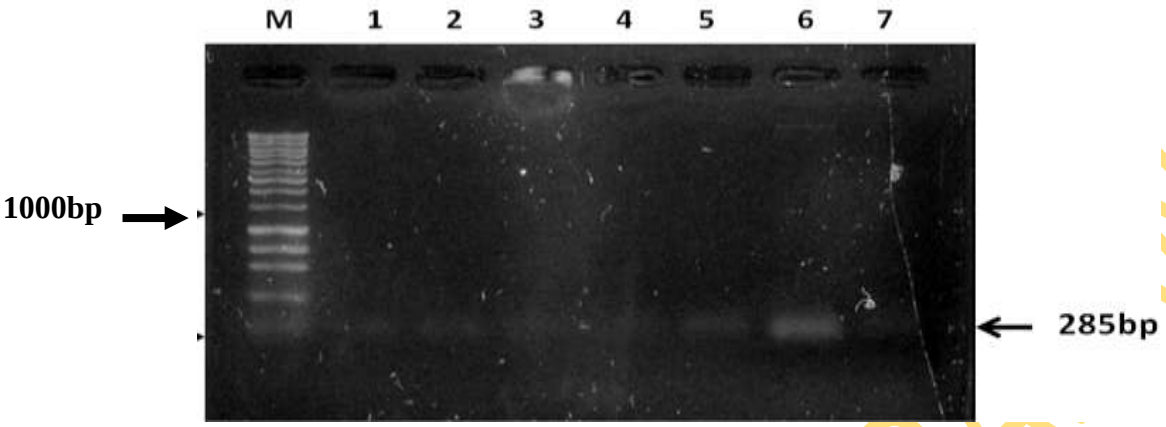

Fig.5.1 Photograph of the 1.5% agarose gel stained with ethidium bromide showing product specific PCR obtained with DNA extracted from kidney samples of cattle naturally infected with leptospire species. Lane M= molecular maker 2kb DNA ladder, Lanes 1 to 7 positive kidney samples of cattle.

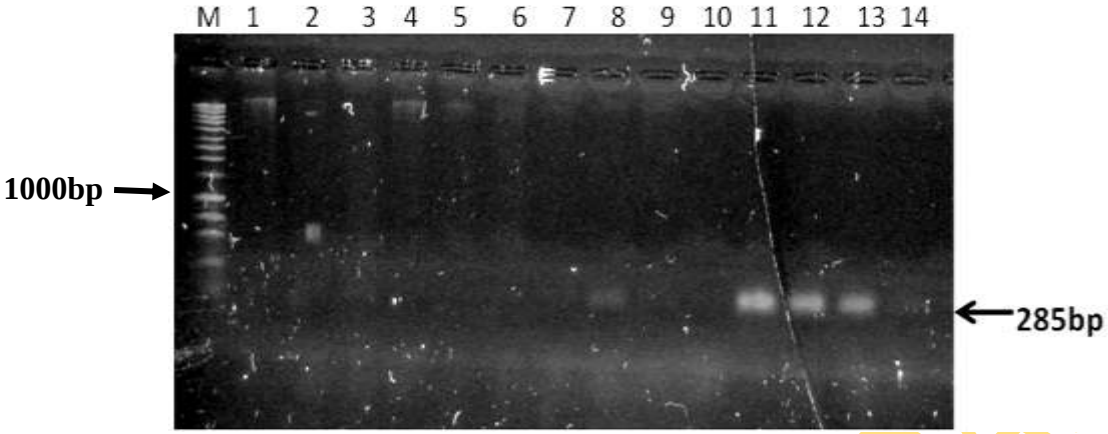

Fig.5.2 Photograph of the 1.5% agarose gel stained with ethidium bromide showing product specific PCR obtained with DNA extracted from kidney samples of cattle naturally infected with leptospire species. Lane M= molecular maker 2kb DNA ladder, Lanes 2, 3, 8, 11, 12 and 13 positive kidney samples of cattle. Lanes 4, 5, 6, 7, 9, 10, and 14, negative kidney samples.

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Table 5.2 Distribution of 108 kidneys collected according to city, Sex and Breeds in both leptospiral positive and negative kidney samples. Data also expressed as a percentage and 95% confidence interval

Cities	Total	(%)	95%CI Lower-upper	Lepto-ve (%)	95%CI Lower-upper	Lepto +ve (%)	95%CI Lower-upper
Ibadan	21	19.4	12 – 26	1 (4.8)	0.0 – 14.0	20 (95.2)	86 – 100
Abeokuta	87	80.6	74 - 88	18 (20.7)	12 – 30.0	69 (79.3)	70 - 88
Sex							
Female	98	90.7	86 - 96	19 (17.6)	11 - 27	79 (88.8)	71 - 87
Male	10	9.3	04 - 14	0 (0)	0.0	10 (9.)	4 – 14
Breeds							
White Fulani	76	70.4	61 - 79	15 (13.9)	07 - 14	61 (68.5)	48 - 66
Red Bororo	9	8.3	03 - 13	0 (0)	0 .0	9 (10.1)	03 - 13
Kuri	4	3.7	0 - 08	4 (3.7)	0.0	0 (0)	0
Muturu	19	17.6	11 - 25	0 (0)	0.0	19 (21.4)	11 - 27

Lepto +ve = leptospiral positive, lepto-ve = leptospiral negative,

5.3.4 Gross Pathological changes in kidneys of cattle which were culturally positive to leptospiral infection.

Table 5.3 depicts the type and distribution of macroscopic changes observed in kidneys from cattle with leptospirosis. Out of the one hundred and eight (n = 108) kidney samples randomly collected from the two abattoirs, only 31 (28.7%) (9 kidneys from Ibadan and 22 from Abeokuta) kidneys showed visible macroscopic changes, while 77 (71.3%) cases appear normal. Leptospiral organisms were isolated from all the 31 kidney samples. The lesions observed on the cortical surfaces of the kidneys include diffuse cortical necrosis (Fig.5.3), icterus, multifocal widespread petechiations and multiple foci of necrosis. Out of the 108 kidney samples, 13 samples (12%, 95%CI from 6 – 18%) showed mild to moderate multifocal areas of necrosis, while moderate multifocal widespread areas of petechial haemorrhages was observed in 9 (8.3%, 95%CI from 3 – 13%) samples. Seven kidney samples (6.5%, 95%CI from 2 – 12%) showed mild icterus and 2 kidney samples (1.9%, 95%CI from 0 – 5%) with moderate diffuse degeneration and necrosis.

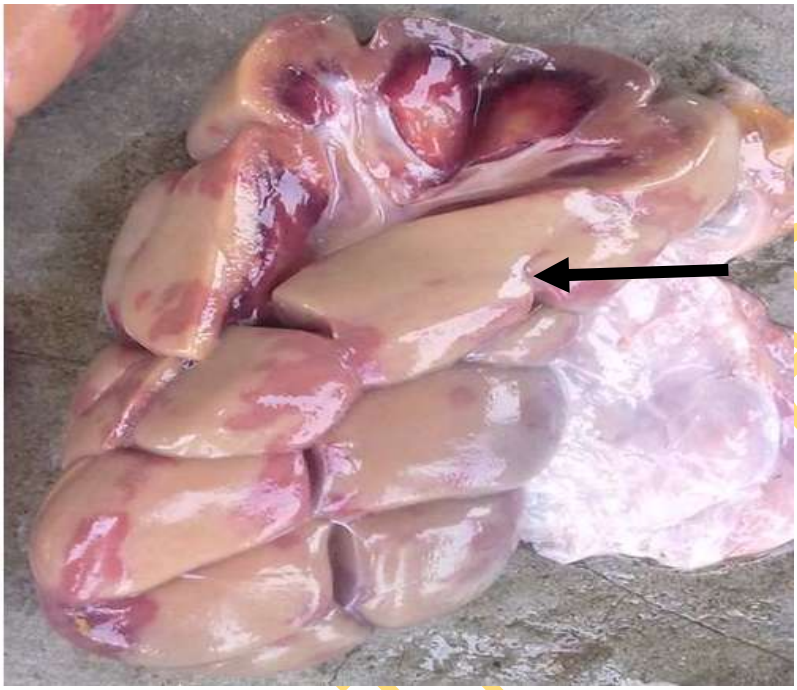

Fig.5.3 Photograph of one of the kidneys showing macroscopic lesion of diffuse cortical necrosis (arrow) in cattle.

Table 5.3 Number and type of macroscopic lesions on the cortical surfaces of the kidneys of cattle. Data also expressed as a percentage and 95% confidence interval

Lesions	No (n=108)	(%)	95%CI Lower	upper
Diffuse nephrosis	2	1.9	0 - 05	
Icterus	7	6.5	2 - 12	
Multifocal petechiations	9	8.3	3 - 13	
Multifocal necrotic areas	13	12.0	6 -18	
No lesions	77	71.3	62 - 80	

Table 5.4 Type and distribution of the macroscopic lesions according to severity on the cortical surfaces of the kidneys

	Degree of the lesions		
Lesions	**+**	**++**	**+++**
Diffuse nephrosis	-	2	-
Multifocal petechiation	2	5	2
Icterus	6	1	-
Multifocal necrotic areas	7	5	1
No lesions	-	-	-

-; Absent, + = mild, ++ = moderate, +++ = marked,

5.3.5 Renal Histopathological findings in both leptospiral positive and negative kidney samples of cattle

Tables 5.5. and 5.6. below depict the renal histopathological alterations and semi-quantification of the lesions observed in both leptospiral positive and negative kidney samples of cattle. Out of the different lesions observed, interstitial lymphoplasmacytic cellular infiltration was the persistent lesion in the kidney samples examined, being observed in 94 (87%) samples out of the 108 kidneys. Out of the 94 (87%, 95%CI from 81 – 93%) samples with interstitial lymphoplasmacytic cellular infiltration (ILPCI), 84 (77.8%, 95%CI from 70 – 86%) kidney samples were positive to leptospiral infection, compared with 10 samples (9.3%, 95%CI from 4 – 14%) that were negative (Figs.5.4-5). The ILPCI was characterised by peritubular, perivascular and periglomerular lymphoplasmacytic cellular infiltration. Ninety–one (84.3%, 95%CI from 77 – 91%) out of the 108 kidney samples showed severe diffuse tubular nephrosis, with 80 kidney samples (74.1%, 95%CI from 66 – 82%) positive to leptospiral infection compared with 11 samples (10.2% 95%CI from 4 – 6%) which were negative to the infection. Eighty-six (79.6%, 95%CI from 72 – 88%) and 85 (78.7% 95%CI from 71 – 87%) kidney samples showed severe tubular dilatation and tubular hyaline casts respectively. Seventy (64.8%, 95%CI from 56 – 74%) out of the 86 showed tubular dilatation compared with 16 (14.8%, 95%CI from 18 – 23%) which were negative, while 64 (59.3%, 95%CI from 42 – 60%) out of the 85 samples demonstrated tubular hyaline cast (Fig.5.6) were positive to leptospiral infection compared with 12 (11.1%, 95%CI from 5 – 17%) kidneys samples which were leptospiral negative. Mild to moderate diffuse tubular vacuolations were observed in 57 (52.8%, 95%CI from 44 – 62%) out of the 108 kidney samples, from which 53 (49.1%, 95%CI from 40 – 58%) kidney samples were positive to leptospiral infection compared with 4 samples that were leptospiral negative.

Moderate focal areas of tubular atrophy were present in 10 (9.3%, 95%CI from 4 – 14%) samples, out of which 7 (6.5%, 95%CI from 2 – 12%) samples were positive to leptospiral infection and the remaining 3 samples (2.8%, 95%CI from 0 – 6%) were negative.

Tubular atrophy was usually observed in areas where there were severe interstitial nephritis and fibrosis. Glomerulonephritis was also observed in 33 (30%, 95%CI from 17 – 43%) kidney samples, out of which 29 (36.9% 95%CI from 11 – 30%) were leptospiral postive and the remaing 4 samples (3.7%, 95%CI from 0 – 8%) were negative. The glomerulonephritis was characterized by mild diffuse membraneous glomerulonephritis (n=12, 11.1%, 95%CI from 5 – 17%) and moderate diffuse membranoproliferative glomerulonephritis (n=21, 19.4%,95%CI from 12 - 26) in both leptospiral positive and negative kidney samples. Other microscopic lesions such as moderate perivascular and interstitial oedema (n= 9, 8.3%, 95%CI from 11 – 27%) and fibrosis (n=53, 49.1%, 95%CI from 40 – 58), mild oxalate crystals (n=4, 3.7%, 95%CI from 0 -8%) and mild tubular calcification were also present in both leptospiral positive and negative kidney samples in this study. Significant difference ($P<0.05$) was observed in the histopathological changes in the kidney of cattle that were leptospiral positive (mean value = 37.27±7.59) compared with those that were negative (mean value = 6.36±1.30). They were also negatively correlated (r = -0.78). Fifty kidney sections that were positive in the EMJH medium were subjected to Warthin Starry silver stain. Thirty seven (74%) out of the fifty samples demonstrated leptospires in their tubules. Some of the sections showed colony of wavy appearance of leptospiral organism within the renal tubules obstructing the tubular lumina (Fig.5.3.15).

Table 5.5 Total numbers of histopathological changes in 108 kidneys of leptospiral positive and negative cattle. Data were also expressed as percentages and 95 percent confidence interval (CI)

Lesions	Total (n=108)	%	95%CI Lower - Upper	lepto +ve (n=87)	(%)	95%CI Lower-upper	lepto –ve (n=21)	(%)	95%CI lower upper
Interstitial lymphoplasmacytic infiltration	94	87.0	81 – 93	84	77.8	70 – 86	10	9.3	04 – 14
Tubular degeneration and necrosis	91	84.3	77- 91	80	74.1	66 – 82	11	10.2	04 – 16
Tubular dilatation	86	79.6	72 – 88	70	64.8	56 – 74	16	14.8	08 – 23
Tubular hyaline casts	85	78.7	71 - 87	64	59.3	50 – 68	12	11.1	05 – 17
Perivascular lymphoplasmacytic infiltration	58	53.7	45 – 63	55	50.9	42 – 60	3	2.8	00 – 06
Tubular epithelial vacuolations	57	52.8	44 – 62	53	49.1	40 – 58	4	3.7	00 – 08
Periglomerular lymphoplasmacytic infiltration	52	48.2	39 – 57	45	41.7	33 – 51	7	6.5	02 – 12
Interstitial fibrosis	53	49.1	40 - 58	41	38.0	29 – 47	12	11.1	05 – 17
Membranoproliferative glomerulonephritis	21	19.4	12 – 26	19	17.6	11 – 25	2	1.9	00 – 05
Glomerular casts	20	18.5	12 - 26	18	16.7	10 – 24	2	1.9	00 – 05
Membraneous glomerulonephritis	12	11.1	05 – 17	10	9.3	04 - 14	2	1.9	00 – 05
Tubular atrophy	10	09.3	04 - 14	7	6.5	02 - 12	3	2.8	00 – 06
Interstitial oedema	9	8.3	11 – 27	9	8.3	03 – 13	-	0.0	00.0
Tubular calcification	5	04.6	01 – 09	2	1.9	00 – 05	3	2.8	00 – 06
Crystal (Oxalate)	4	03.7	00 – 08	2	1.9	00 – 05	2	1.9	00 – 05

Lepto+ve: leptospire positive, Lepto –ve: leptospira negative.

Table 5.6 Semi-quantification of the degree of histopathological lesions in the 108 kidneys of leptospiral positive and negative cattle.

Lesions	**Lepto positive**	**Lepto negative**
Interstitial and perivascular oedema	++	-
Tubular degeneration and necrosis	+++	+++
Tubular epithelial vacuolations	++	+
Interstitial fibrosis	++	+
Interstitial lymphoplasmacytic	+++	+
Perivascular lymphoplasmacytic	+++	+
Periglomerular lymphoplasmacytic	++	+
Glomerular atrophy	+	+
Tubular hyaline casts	+++	++
Glomerular casts	++	+
Tubular dilatation	+++	++
Membraneous glomerulonephritis	+	++
Membranoproliferative glomerulonephritis	++	++
Crystals (oxalate)	+	+
Tubular calcification	+	++
Tubular atrophy	++	+

-; Absent, + = mild, ++ = moderate, +++ = marked, Lepto+ve =Leptospire positive; Lepto-ve = Leptospire negative

Intact leptospiral organism were observed in some tubules. In some cases, whole leptospiral colony were observed freely (Fig.5.7a & b) or attached within the tubular lumina, while some were embedded within the protein hyaline cast.

The immunohistochemical staining of the sections showed positive reactivity with 6 sections used in this study. Reactivity was observed with serovars hardjo (4) and pomona (2). (Figs.5.8a & b) Leptospire antigens were present within the renal tubules, mostly within the tubular epithelia cytoplasms, and on the apical surfaces of the tubular epithelial cells.

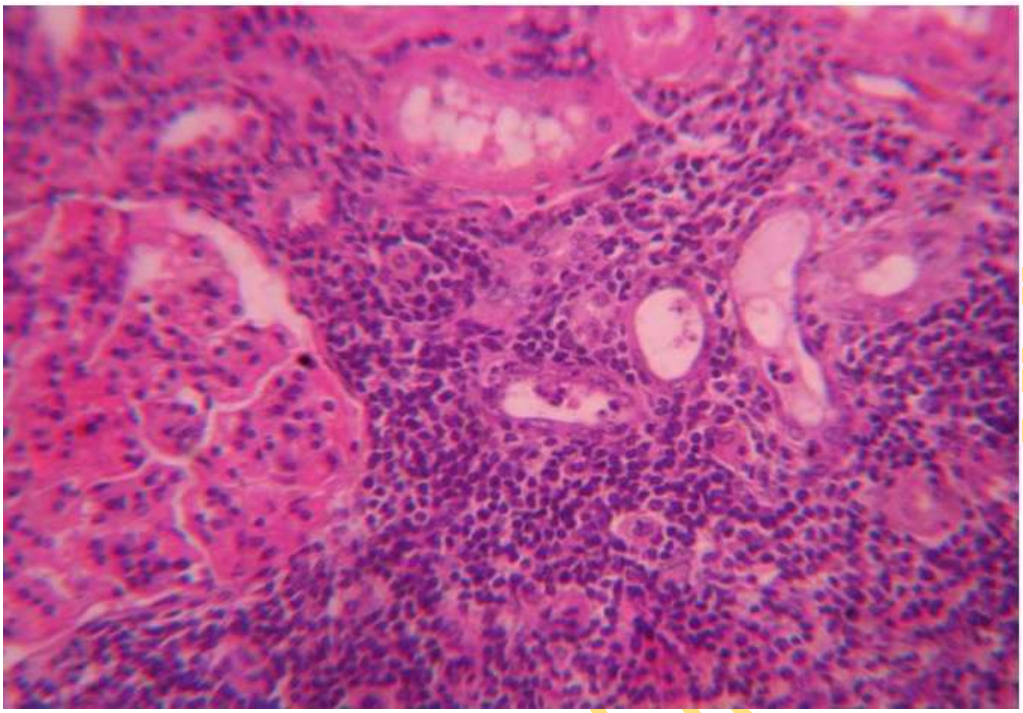

Fig.5.4 Kidneys showing severe tubular degeneration and necrosis, tubular atrophy and effacement due to marked diffuse interstitial and periglomerular mononuclear cellular infiltratione. H&E. x 350

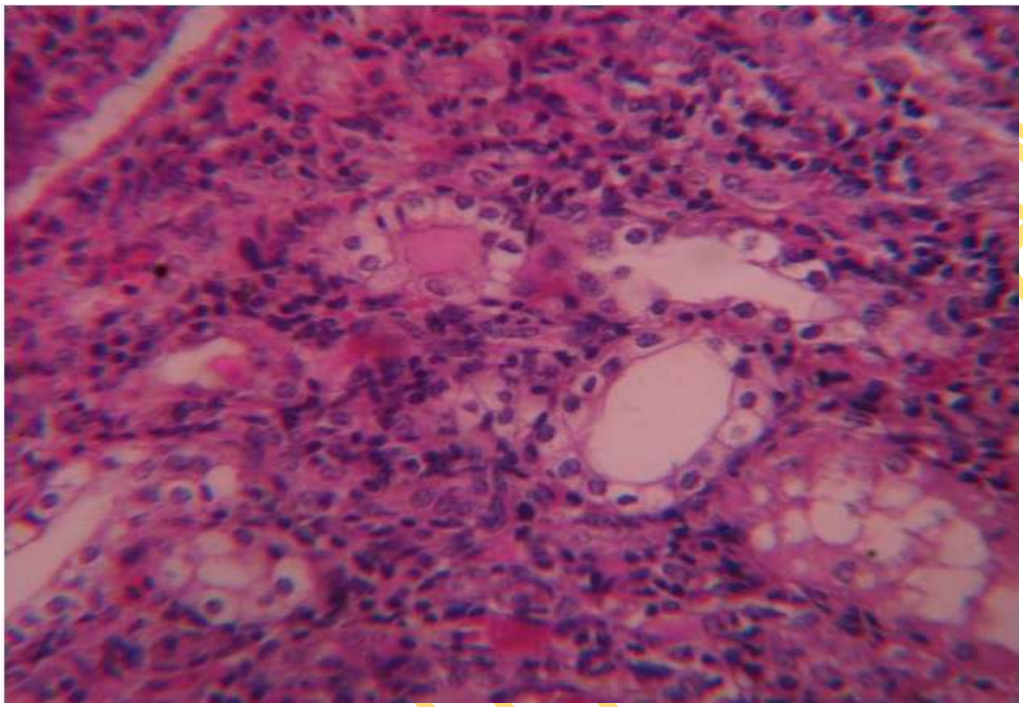

Fig.5.5 Photomicrograph of the kidney showing diffuse tubular epithelial vacuolations and moderate diffuse interstitial mononuclear cellular infiltrations, H & E. x 350.

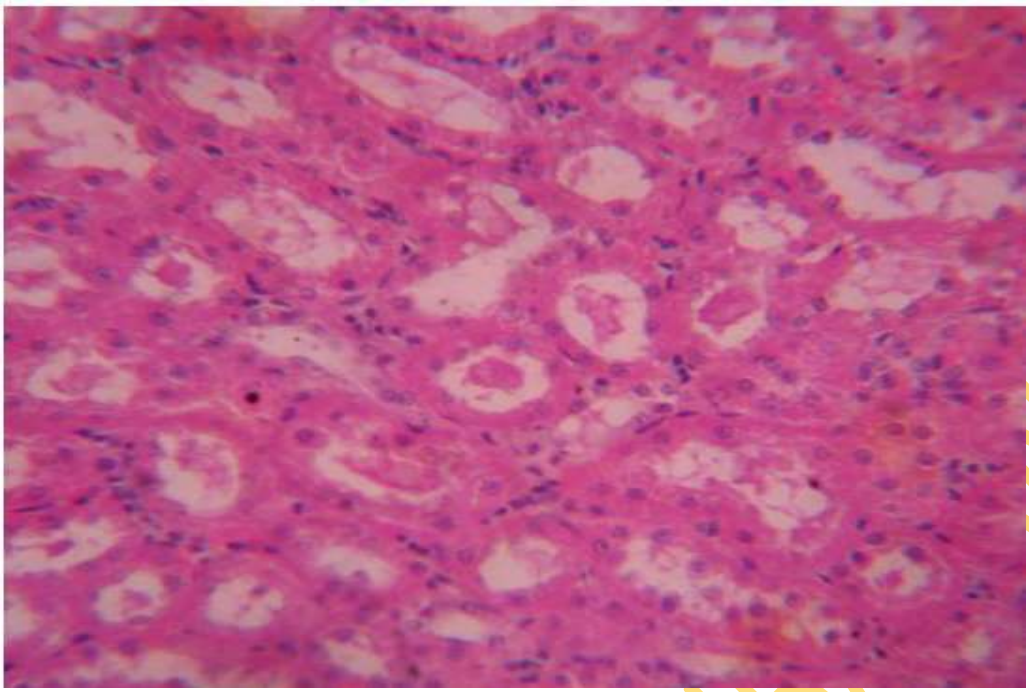

Fig.5.6 Photograph of the kidney showing moderate tubular degeneration and necrosis with deeply oesinophilic proteinacious cast in the tubular lumina. H & E. X 300,

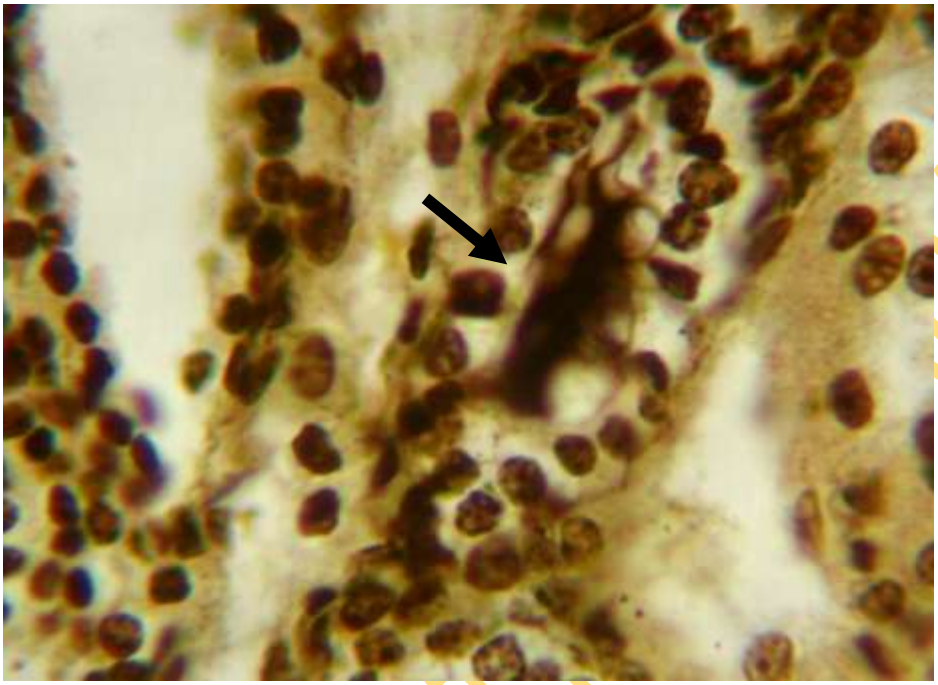

Fig.5.7 Photomicrograph of the kidneys showing the wavy appearance of the leptospiral organism (arrows) in the tubular lumen of the medulla Warthin Starry silver stain. x 450.

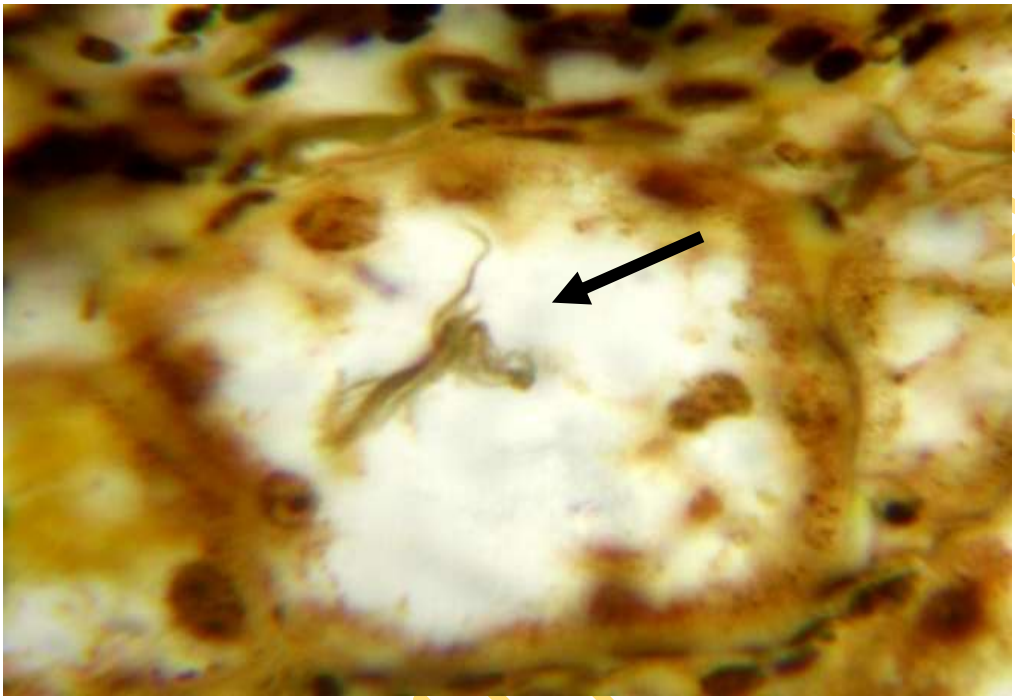

Fig.5. 8 Photomicrograph of the kidneys showing free leptospiral organism in the tubular lumen of distal convoluted tubule. Warthin Starry silver stain . x 400

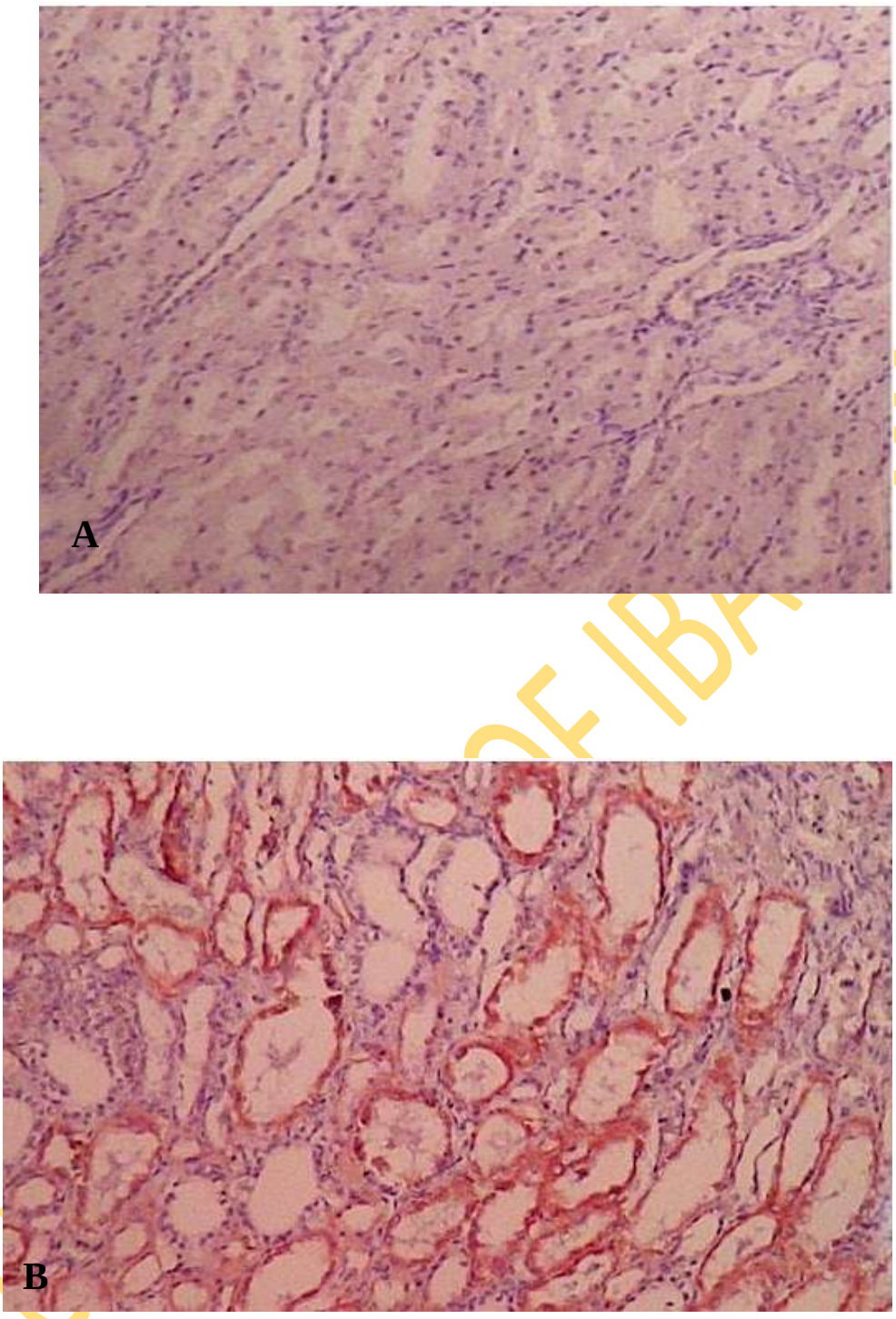

Fig. 5.9a & b Kidney section of cattle showing normal (A) and severe *Leptospira hardjo* colonization of the tubules of the corticomedullary junction (B) (Streptavidin-biotin immunoperoxidase method, counterstained with Mayer's haematoxylin) X 250

Table 5.7 Comparative summary of different leptospiral serovars in dogs, cattle and wild rats characterized by monoclonal antibodies.

		Dogs (n=27)		Cattle (n=31)		Wild rats (n=17)		
	Serotypes	No positive	(%)	No positive	(%)	No positive	(%)	Agglutination titres
1	*L. Pomona*	5	18.5	1	3.20	5	23.8	1: 3200
2	*L. Grippotyphosa*	4	14.8	3	9.70	3	14.3	1: 3200
3	*L. Hardjo*	-	-	9	29.0	-	-	1: 400
4	*L. Bratislava*	3	11.1	7	22.6	1	4.8	1: 1600
5	*L. Canicola*	4	14.8	2	6.50	3	14.3	1: 800
6	*L. Icterohaemorrhagiae*	8	29.7	1	3.20	7	33.0	1: 1600
7	*Undetermined serovars*	3	11.1	6	19.4	2	9.5	-

Table 5.8 Comparative summary of methods of detection for the presence of leptospira organisms in kidneys of cattle

	Number positive/number examined													
Tissue	Gross	%	Histopath	%	MAT	%	EMJH	%	WSSS	%	PCR	%	IHC	%
Kidney	31/108	28.7	84/108	77.8	25/31	80.7	89/108	82.4	37/50	74	13/21	61.9	6/6	100

WSSS, Warthin Starry silver stain, MAT; microscopic agglutination test, PCR; Polymerase chain reaction,; IHC; immunohistochemistry, EMJH : Ellinghauson-McColloughsen Johnson – Harris medium

5.4 DISCUSSION

Leptospirosis was diagnosed in 89 out of 108 kidney samples collected from the two abattoirs in southwestern Nigeria. The diagnosis of leptospira infection was based on one or more of the following: organ gross and histopathological changes, cultural isolation using EMJH medium at 27^{o}C-30^{o}C and dark field microscopy, polymerase chain reaction (PCR), microscopic agglutination test using monoclonal antibodies (MAT) and demonstration of typical spirochetes in the kidney samples by Warthing-Starry technique and immunohistochemistry.

Leptospirosis can be diagnosed by several laboratory methods, of which the serological methods are the most commonly used, but the isolation allows the definite diagnosis of individual infections. In Nigeria, there are few works on the isolation of leptospires from animals, as the majority of the data is limited to serology. This study appeared to be one of the most comprehensive studies in the diagnosis of pathogenic leptospires in cattle in the continent of Africa, since most of the works available were base on serology, little cultural isolation and the use of polymerase chain reaction (Mgode *et al.*, 2006; Ralinirina *et al*., 2010).

It has been reported that leptospirosis can be transmitted venerally and can persist in the genital tract in infected cattle (Cynthia *et al*, 2005) but the significance of the disease as it relates to gender and breeds susceptibility is not known. However, the data gathered indicates that females were more affected than males in this study. Although, most of the kidney samples collected from male animals were positive for pathogenic *leptospira interrogans*, but kidney samples collected from the female were more than the male animals. This might also be due to leptospiral-related reproductive disease which made the farmers to cull them.

The importance or the significance of cattle breeds in the epidemiology of leptospirosis is not understood. Four breeds of cattle were studied from both abattoirs which

include White Fulani, Red bororo, Kuri and Muturu. White Fulani had the highest percentage of positive cases compared with other breeds examined. The higher percentage of positive cases in the White Fulani in this study might probably be due to the large number of these cattle in the southwestern state above other breeds or that they were the most susceptible breed of cattle in this country. This was followed by Muturu and then Red bororo. Interestingly, none of the four kidney samples collected from the Kuri breed tested positive for leptospiral infection. Although the reason for this is unknown, but it is possible to speculate that this might have been due to (1) previous vaccination of the breed which could have resulted in increased antibody titre (2) the small number of kidney samples collected from the breed and (3) because they are resistant to leptospiral infection. These assertions might need further investigations.

The prevalence of leptospiral organism in cattle population in this study might not be a true reflection of the disease in the whole country, because of the limited number of cattle involved and the diverse origin of the animals. Nevertheless, it enables us to obtain a safe record of leptospiral infection in cattle that are being slaughtered in these abattoirs. It also enables us to know which leptospiral serovar is more prevalent in cattle and the possible serovars people and domestic animals might likely have contact with. It can be inferred that 82.4% of a total of 108 cows being slaughtered in these two abattoirs were infected by Leptospira spp. This infection is likely a reflection of natural exposure, since cattle vaccination is not being practiced in Nigeria (Agbogu *et al.*, 2006).

Different leptospiral serovars have been isolated from cattle urine and kidneys in various part of the world (Heer, 1982; Feresu, 1992). Cattle are considered maintenance hosts of the serovar hardjo (Moreira *et al.*, 2004). Serovar hardjo has two distinct genotypes: hardjobovis and Hardjoprajitno. The genotype hardjobovis belongs to the species *Leptospira borgpetersenii,* while the genotype hardjoprajitno belongs to the species *L. interrogans*. Both

are important causes of reproductive disorders in cattle herds worldwide and have different clinical manifestations (Faine, 1999). In this study, *leptospire* interrogan *hardjoprajitno* was the predominant serovar with 9 (29%) out of the 31 leptospiral isolates. This is closely followed by serovar bratislava with 7 (22.6%). This is in agreement with the works of Mineiro *et al.,* (2011) in Brazil in which serovar bratislava was second to serovar hardjo in prevalence in cattle. The most commonly encountered serovar in northern Spain is bratislava, followed by the serovar hardjo (Atxaerandio *et al.*, 2005). Serovar icterohaemorrhagiae and grippotyphosa were 3 (9.70%) each which might possibly be responsible for the macroscopic changes such as icterus and haemorrhages observed in the affected kidneys in this study (Mgode *et al.,* 2006; Ralinirina *et al*., 2010). Serovar canicola and pomona were 2 and 1 isolates respectively. The isolation of serovar canicola from the kidney of cattle in this study appeared to be an unexpected finding in cattle, since dogs are worldwide recognized as maintenance host. This is in agreement with the works of Ezeh *et al.,* (1996) who serologically detected the antibodies of canicola in 9.5% of 1537 cattle examined, and the works of Zacarias *et al.,* (2008) who isolated canicola in the kidneys of cattle in Brazil. The source of canicola infection in this study is unknown, but it is possible to suggest that this might be from the sheperd dogs that follow cattle rearers in this part of the world. The isolation of different serovars of leptospire in this study is in agreement with the studies of Ezeh *et al.,* (1996), who serologically showed the prevalence of different leptospire serovars from the sera of 1,537 cattle, using MAT in Jos, Nigeria. In their study, leptospire hardjo was 35.6 %, pomona 11.7%, pyrogenes 11.7%, canicola 9.5%, grippotyphosa 7.7%, bratislava 5.9%, icterohaemorrhagiae 5.9 %, ballum 4.5%, autumnalis 3.6%, bataviae 2.3% and tarassovi 1.8%. Moreover, Agunloye (2002) also evaluated the prevalence of leptospiral antibodies in sheep and goats by MAT using a total of seven leptospiral serovars. Out of the 575 animals tested, 17.7% were positive to leptospira by the MAT. The prevalence in sheep

and goats was 23.5% and 13.1%, respectively. The highest reacting leptospira in both species was *L. pomona* with a rate of 25.9%. This was followed by *L.icterohaemorhagiae* (17.9%) and *L. autumnalis* (17.0%), respectively. Although Ezeh *et al.*, (1989) isolated serovars hardjo, pyrogenes and the new serovar *nigeria* from cattle slaughtered at Jos abattoir in Nigeria and Diallo and Dennis (1982) also isolated strains of serovar *pyrogenes* from kidneys of cattle in Zaria, Nigeria, but there was no report of isolation of serovars such as icterohaemorrhagiae, gripptotyphosa, bratislava and canicola in their studies as it is documented in this study. Thus, it is possible to infer that exposure of both humans and animals to these serovars is possible, especially if cattle are slaughtered during the leptospiremic phase of the infection or through meats that are not properly cooked as suggested by Okewole and Ayoola (2009) and Barr, (2002).

The PCR revealed the presence of pathogenic leptospire interrogans amplicons in 13 (61.9%) out of the 21 samples examined from naturally infected cattle in this study. PCR has been shown to be an effective diagnostic method for leptospirosis in domestic animals (VanEys *et al.*, 1989) and a possible replacement of the old methods such as MAT and culture isolation because of their long or delay process. This study is in agreement with these workers considering 61.9% (13 samples) out of the 21 samples examined. The remaining 8 (38.1%) negative samples in which there was no amplification might be due to intrinsic factors in the culture isolates or they do not belong to the genomospices leptospire interrogans in which primers G1/G2 can only amplify their DNA. Pathogenic *leptospire kischneri* in which gripptotyphosa belong with a different primer might possibly have amplified DNA from the remaining 8 samples.

The gross renal lesions, such as cortical haemorrhage, multifocal areas of necrosis, diffuse palor and icterus, reported in this study were typical macroscopic lesions of renal bovine leptospirosis and they were consistent with those previously reported in cattle and

other animals (Faine *et al.*, 1999). In this study, there was no significant correlation between gross lesions and isolation of leptospiral organism in the kidney samples, since most of the kidney samples without gross lesions were culturally positive and the leptospiral organism demonstrated in the renal tubular lumina.

The main histological changes observed in bovine leptospirosis are observable in the kidneys (Francielle *et al.*, 2005), but Skilbeck *et al.*, (1988) did not observe significant lesions in kidneys from which leptospires were isolated. However, in this study, the lesions range from mild to moderate extensive inflammatory infiltrates (mostly lymphocyte and plasma cells) to diffuse lesions, characterized by tubular necrosis, glomerular atrophy and renal haemorrhage. Most of the studied kidneys samples presented histopathological changes suggestive of leptospirosis in accordance with the works of Faine *et al.*, (1999). Interstitial fibrosis might have been due to the subsiding interstitial inflammatory reaction induced by the leptospiral organism with decreasing inflammatory cells and subsequent scarification of the lesion.

The methodologies used in this study were found to be sensitive enough for the diagnosis of leptospirosis in kidney samples, but they were more sensitive than the others. Considering the number of samples used in each methodology, Warthin Starry silver stain was 75% in sensitivity and 100% in specificity, PCR showed 61.9% sensitivity and specificity of 100% while immunohistochemistry was 100% in sensitivity and specificity. In this study leptospire diagnosis were made with silver staining only by the presence of long wavy spirochetes in the tissue sections, as reported previously (Scanziani *et al.*, 1989; Yener and keles, 2001). It was impossible to make diagnosis of leptospirosis of other forms such as short rods, aggregates, or cocci by Warthin Starry silver stain because they were not distinguishable from artifacts except when the organism are located in an hollow organ or structure. Additionally, the IHC technique had several advantages over silver staining: 1) it

enabled the specific demonstration of leptospires together with light microscopic changes in tissue sections, 2) leptospires could be demonstrated not only as whole bacteria but also as intra- and extracellular granules, 3) the samples could be easily evaluated on low magnification because of the good contrast of the stained leptospiral antigens over the blue background staining as stated by (Szeredi and Haake 2006). Although, IHC can be used as a rapid screening procedure in the diagnosis of diseases, but it is not cost effective and many arrays of monoclonal antibodies would be needed compare with Warthin Starry silver stain.

Serovar identification is important for epidemiologic studies and for potential development of a vaccine for cattle leptospirosis (Donahue and Williams, 2000). Some authors have suggested the use of serovar specific monoclonal antibodies on tissue sections for serovar identification (Szeredi and Haake 2006), but the use of monoclonal antibodies and immunohistochemistry to characterize leptospiral organism in this study have shown the different serovars in cattle population being slaughtered in abattoirs of the studied areas. It also showed the public health implications and the zoonotic importance of the serovars both in humans and domestic animals, especially dogs when fed with improperly cooked meats or feotus of cattle during leptospiremic phase of the infection.

CHAPTER SIX

6.0. PATHOGENESIS AND PATHOLOGY OF EXPERIMENTAL LEPTOSPIRAL INFECTION IN GUINEA PIGS

6.1. INTRODUCTION

Clinical leptospirosis is characterized by broad non-specific clinical manifestations, ranging from subclinical infection to a severe fatal disease, characterized by wide spread serosal petechial and ecchymotic hemorrhages, acute renal failure and jaundice (Yang *et al.*, 2006). Although, major progress has been made in elucidiating the pathogenesis of the disease, but much needed to be known on the organ and tissue distribution with the pathological effects excerted by this organism in the various tissues.

Leptospires are highly motile bacteria that are able to penetrate skin and mucous membrane, and rapidly disseminate to other tissues shortly after infection. In susceptible hosts such as humans, systemic infection can produce severe multi-organ manifestations, including jaundice, acute renal failure and severe haemorrhage in the lungs and other organs. However, in animal reservoirs such as the domestic rat, infection produces chronic and persistent asymptomatic carriage in the renal tubules (Levett, 2001; Bharti *et al*., 2003; McBride *et al.*, 2005).

Haemorrhage has been the most consistent gross morphological changes in both natural and experimental leptospirosis in the literature (Faine, 1957; Yang *et al.*, 2006). According to the report of Faine, (1957) in guinea pigs infected with serovar icterohaemorrhagiae, haemorrhage has been the most consistent and prominent gross morphological lesion observed in the abdominal wall, lung and retroperitoneal fats.

The occurrence of thrombocytopenia in guinea pigs infected with pathogenic leptospires abounds in the literature. However, its relationship with disseminated intravascular coagulation (DIC) remains controversial. In one haematological study, thrombocytopenia

found in guinea pigs did not correlate with the occurrence of DIC, but the platelet aggregation and Kupffer cells phagocytosis were suggested as the potential causes of thrombocytopenia (Yang, *et al.*, 2006).

The use of experimental models of leptospirosis remains a critical component for elucidating disease pathogenesis. Although mice are usually not susceptible to leptospirosis, but various reports have described lethal infections in young mice, which carry a mutation inactivating the *trl4* gene (Pereira *et al.*, 1998; Nally *et al.*, 2005), and also in cyclophosphamide-treated mice (Masuzawa *et al.*, 1991). Like rats, the common domestic mouse is an asymptomatic carrier of pathogenic leptospires. Most studies exploring the pathogenicity of leptospire have therefore used hamsters or guinea pigs as animal models. In these species, infection with virulent leptospire causes a fatal acute disease similar to severe human and animal disease (Levett, 2001; Bharti *et al.*, 2003; McBride *et al.*, 2005). Other animals have been used for modelling leptospirosis, including gerbils (Yukawa *et al.*, 1990; Sonrier *et al.*, 2000) and marmoset monkeys (Pereira *et al*., 2005).

Intra-peritoneal route of leptospiral infection produces a lethal infection in guinea pigs and mimics the clinical presentation of severe leptospirosis in animals and humans. However, this route of infection may not reflect conditions encountered during natural infection, as leptospires enter the host by either through oral or mucus membranes or abraided skin.

Different workers have used polymerase chain reaction, immunohistochemistry, Warthin Starry silver stain and isolation with EMJH techniques (either solely or in combination of any of the two methodologies) to detect pathogenic leptospires in infected animals and humans (Smythe *et al*., 2002; Truccolo *et al.*, 2002; Segura *et al*., 2005; Viriyakosol *et al.*, 2006; Roczek *et al.*, 2008), yet, little knowledge has been demonstrated on the dissemination of leptospiral organism in the tissues and the time of leptospiral invasion and disappearance in different organs.

In this study, different approaches, including polymerase chain reaction, immunohistochemistry, Warthin Starry silver stain and isolation with EMJH techniques would be used to gain a better insight into leptospiral disease pathophysiology in the guinea pig infection model in a time-related assessment of different organs. The combination of these different methodologies might proved to be effective in determination of the efficacy of antibacterial drugs (Truccolo *et al.*, 2002), the assessment of protective immunity of vaccine candidates, determination of the tissue distribution of different pathogenic serotypes in this guinea pig infection model of leptospirosis and determination of the best approach in the management of the disease during the course of canine leptospirosis.

Therefore the objective of this study was to (i) determine whether leptospiral isolate from dog in infected guinea pigs would mimic the pathological changes observed in dogs, (ii) determine the dissemination kinectics of leptospiral organism in tissues and different organs in the infected guinea pigs, (iii) evaluate the haematological response in the leptospiral infected guinea pigs, (vi) determine the role of the oxidative stress (lipid peroxidation) in the pathogenesis of aneamia in the infected guinea, (v) determine the changes in some biochemical parameters during leptospiral infection in guinea pigs, (vi) determine the underlying histopathology in different organs of infected guinea pigs and compare them with those observed in dogs.

In this study, these aims and objectives would be realized through different approaches which include isolation with EMJH medium, polymerase chain reaction, Warthin Starry silver stain and immunohistochemistry to gain a better insight into leptospiral disease pathophysiology in organs such as the brain, spleen, kidney, lung, heart, adrenal gland, pancreas and the liver of the guinea pig infection model.

6.2 MATERIALS AND METHODS

6.2.1 Source of the leptospiral Isolate

The leptospires isolate used for this study was obtained from the kidney and blood of 3 naturally infected dogs. The carcasses of the dogs were presented to the Veterinary Teaching Hospital, University of Agriculture Abeokuta, Nigeria for postmortem examination. The dogs had history of anorexia, emaciation and dehydration with subcutaneous jaundice and vomiting with gross pathology of pulmonary, renal and intestinal haemorrhages. Kidney of the dog was macerated and inoculated into EMJH medium as described above (section 5.2.3). The organism was identified under dark field microscopy.

6.2.2 Determination of the serovar of leptospiral isolate.

Microscopic agglutination test (MAT) was carried out to determine the serotype of the leptospiral isolate form the dogs. The whole set of *L. canicola*, *L. icterohaemorrhagiae*, *L. Pomona*, *L. hardjo*, *L. bratislavia* and *L. grippothyphosa* monoclonal antibodies were kindly offered by the Royal Tropical Institute, Amsterdam, Netherlands. Serial dilution of rabbit monoclonal antibodies were made in phosphate buffered saline (PBS) pH 7.2 and 50 µL aliquots placed in each well of a microtitre plates to which was added an equal volume of a 7-10 days old culture of live leptospires in EMJH medium. After 2-4 h of incubation at 37 ºC, the agglutination of the isolate with the exact monoclonal antibody was determined by dark field microscopy. The monoclonal antibody that immobilized 50% of the free moving leptospiral organism under the dark-field microscope was considered the exact serovar.

6.2.3: Determination of pathogenic status and virulence of the leptospiral isolate

The virulence nature of the isolate was determined in laboratory animals. A group of four guinea pigs (Approximately 150 g) were inoculated intraperitoneally with 1.0 mL of EMJH medium containing 10^7 leptospires. The leptospire counting in the EMJH medium was performed under dark field microscopy (Faine, 1999), and the inoculum with 10-20 bacteria per 200x microscopic field was used. Animals were confined in separate cages and monitored daily for the presence of clinical signs and symptoms. The re-isolation was performed from the kidney tissues of the inoculated guinea pigs for confirmation.

6.2.4 Experimental Animals

Sixty guinea pigs weighing between 150 to 200 grams were used for the whole study. These were divided into two groups of 30 animals each. They were purchased from the local market in Ibadan and brought to the Department of Veterinary Pathology University of Agriculture, Abeokuta. They were caged in a fly-proof animal house and maintained on standard diets. Drinking water was provided *ad libitum.*

6.2.5 Experimental Design

The 60 guinea pigs were divided into 2 groups A and B of 30 animals in each group. Group A animals were used for the haematological and oxidative stress studies while the animals in group B were used for dissemination kinectic studies and the pathology of the various organs. The experiments were approved by the animal research committee of the Faculty of Veterinary Medicine University of Ibadan.

6.2.6 Experimental infection

Twenty guinea pigs were inoculated intraperitoneally with 1 ml of the leptospiral culture (1×10^7) as previously described by Fanie, (1999) while the remaining ten guinea pigs were given EMJH medium intraperitoneally as the negative controls.

6.2.7 Blood sample collection

Blood samples were collected from the group A at 12hrs, 1, 2, 3, 4, and 5 days post infection by cardiac puncture from 5 guinea pigs (3 from the infected group and 2 from the control group). The blood samples were collected in sample bottles containing ethylene-diaminetetraacetic acid (EDTA) anticoagulant. Sera were obtained from the blood samples collected in the plain bottles without anticoagulant.

6.2.8 Determination of haematological and serum biochemical parameters.

Haematological parameters were determined using Jain's method (1976). Packed cell volume (PCV), Haemoglobin (Hb) concentration, Red Blood Cell (RBC). Reticulocyte count was also determined according to Schalm *et al.*, (1975). Erythrocytes indices (MCV, MCHC), White blood cell count (Total and differential), platelet counts were also determined using standard methods.

6.2.9 Determination of Oxidative stress (lipid peroxidation) in guinea pigs experimentally-infected with *leptospira interrogans* serovar *icterohaemorrhagiae*

6.2.9.1 In vivo plasma lipid peroxidation in guiea pigs experimentally-infected with *Leptospira interrogans* serovar *icterohaemorrhagiae*

Plasma was obtained from the heparinized blood by centrifugation at 2000 x g room temperature at 12hrs, day 1, 2, 3, 4 and 5 p.i, Briefly, 0.05ml of plasma (distilled water for blank) was added to 1.0ml thiobarbituric acid (TBA). The mixture was then incubated at 10-0^0C for 15minutes in a water bath. The test tubes were then put in cold water to stop the reaction. After cooling to room temperature, the mixtures were centrifuged at 1000rpm for 10mins and the TBARS (thiobarbituric acid reactive substances) of the clear supernatant was read spectrophotometrically at 535nm (Buege and Aust, 1978).

6.2.9.2 In vitro erythrocyte peroxidation in guinea pigs experimentally-infected with *Leptospira interrogans* serovar *icterohaemorrhagiae*

The in vitro lipid peroxidation of erythrocytes was measured according to the method described by Igbokwe *et al.,* (1994). Approximately one trillion (10^9) washed erythrocytes per animal was obtained from the middle third of packed red cells after centrifugation. The in-vitro erythrocyte peroxidation assay was carried out on 12hrs, days 1, 2, 3, 4 and 5 post infections. The washed erythrocytes were briefly suspended in 2ml of 0.9% normal saline in labeled sample tubes. To each of these tubes was added 0.5ml of 1.5% hydrogen peroxide, while washed erythrocytes suspended in 2.5ml of 0.9% normal saline served as the control. The samples were incubated at 37^0C for 90 minutes after which the reaction was stopped with 0.5ml of 10% trichloroacetic acid. The suspension was centrifuged at 1500g for 10 minutes followed by filtration through Whatman No. 1 filter paper. To each filtrate was added 0.75ml of 0.67% thiobarbituric acid and then re-heated at 100^oC for 20 minutes. The samples were cooled to 20^oC and the by-products of peroxidation (TBARS) were measured spectrophotometrically at 535nm.

6.2.10 Determination of Biochemical Parameters

Hepatic enzymes concentration such as alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) were determined through spectrophotometer using commercially available kits (Randox Laboratories Ltd. Crumlin, UK). Total plasma protein, Albumin, and globulin were determined according to the method of Gornall *et al.,* (1949) using Randox diagnostic reagent kit. The determination of bilirubin was carried out as described by Jendrassik and Groff (1938) using Randox diagnostic reagent. Blood urea nitrogen (BUN) and creatinine were also determined Serum electrolytes levels such as; Na, K, Cl, and H_2CO_3 were determined using Randox diagnostic reagent kits.

6.2. 11. DNA Extraction

DNA was extracted from the pure cultures of leptospiral organism isolated from liver, kidney, spleen, heart, pancreas, adrenal gland, lung and the brain of the infected guinea pigs. Genomic DNA was extracted from the cultures isolates by the method described by Boom *et al.,* (1990), using a commercial available DNA extraction kit (Quick-gDNA^{TM} Microprep, Zymo research, USA).

6.2.12 Primers

The primers used were G1 (5'-CTGAATCGCTGTATAAAAGT-3') G2 (5'-GGAAAACA AATGGTCGGAAG-3'). These primers are traditionally known to be diagnostic of leptospirosis and were described by Gravekamp *et al.* (1993). PCR amplification was carried out in a total volume of 25µL. Amplification was processed in a thermocycler with an initial cycle of 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 51°C for 1 min and 72°C for 1 min, with a final extension of 72°C for 7 min. The PCR product was analyzed by electrophoresis in 1.5% agarose gel, stained with ethidium bromide (0.5 mg/ml) and visualized by UV trans-illuminator

6.2.13 Pathology of leptospirosis in different organs of infected guinea pigs

The remaining tissues were fixed in 10% buffered formalin after gross morphological observations. The tissue samples were processed routinely using haematoxylin and eosins stains and Silver Staining according to Young's modification of Warthin and Starry (1920) was used in identification of the Leptospires organism in the tubular lumina of the cattles. The degree of the histopathological lesions in the organs sections were recorded as +++ = severe or marked, ++ = moderate + = mild and - = absent.

6.2.14. Immunohistochemistry

Serial 5-µm sections from each paraffin block were mounted on electrically charged slides and allowed to dry overnight. The sections were deparaffinized in 4 changes of xylene for 3 min each, rehydrated in a graded series of ethanol solutions (100%, 95%, 70%, 50%), and finally washed with deionized water. The deparaffinized sections were then placed in a Coplin jar containing 0.01 M sodium citrate buffer (pH 6.0) and heated for 40-mins periods, followed by cooling to room temperature for 20 mins, to unmask the antigens.

Following 5 min of washing in 0.05 M Phosphate-buffered saline (PBS) solution (pH 7.6) containing 0.05% Tween 20 (TPBS). All steps were done at room temperature.

The sections were first incubated in 3% hydrogen peroxide for 15 min to quench endogenous peroxidase. After a brief wash of TPBS, nonspecific binding was blocked by bathing in normal goat serum (1:10 dilution in PBS) for 10 min. The tissues were then incubated with rabbit monoclonal (1:800 dilution in PBS) for 30 min. After a brief wash with TPBS, the slides were incubated with biotinylated secondary antibody (goat anti-rabbit IgG) for 15 min.

After a last wash in TPBS, the slides were incubated with Alkaline phosphatase for 15 min. Slides were then rinsed with distilled water and incubated with chromogen for 10 min. Slides were finally rinse with distilled water to stop the staining process. Sections were counterstained with Mayer haematoxylin for 3 min followed by a 5-min rinse in running tap water and were mounted with glycerol water-soluble mounting medium for microscopic evaluation. Negative controls were slides from Leptospira-infected tissues that were subjected to the same staining procedure as the others except that normal rabbit serum was used in place of the monoclonal antibody.

6.2.15 Dissemination kinetics and pathology of the organs in leptospiral infected guinea pigs using EMJH medium, PCR and immunohistochemistry

Six guinea pigs (4 infected and 2 uninfected controls) were euthanized at different time interval by ether and were aseptically opened. About 0.3-0.5g of tissue from different organs such as liver, kidney, spleen, heart, pancreas, adrenal gland, lung and the brain were taken for cultural isolation and PCR at different time of 12 hrs, day 1, 3, 5, and 7 post infections.

Parts of the organs were fixed in 10% buffered formalin for histological studies. Special staining such as Warthin Starry silver stain was performed from the cut sections of different organs. Immunohistochemistry was performed on various organs as described in section 4.2.6.1 above. The dissemination kinectics of leptospiral organism using immunohistochemistry slides of 2 guinea pigs was carried out from the 4 guinea pigs euthanized at different intervals. Intact or granular leptospiral organism stained immunohistochemically within the tissues and mononuclear cells were counted at X 250 per field with the aid of Olympus light microscope. The organs examined include; spleen, liver, kidney, brain and lung according to the method of Merien *et al.*, (1998).

6.3 RESULTS

6.3.1 Morphology and staining

The isolate used in this experimental aspect showed typical morphology and characteristic motility of the genus leptospira under dark-field microscopic examination (Fig.6.1). The organism was well stained by silver impregnation technique. Leptospires were stained brownish to dark-brown on a light-to-golden background.

6.3.2 Determination of leptospiral serovar isolated from dogs

Out of the six monoclonal antibodies used in the determination of the serovar of the isolate, monoclonal antibody *leptospira icterohaemorrhagiae* was observed to agglutinate more than 50% of the isolate. This isolate was used in the experimental infection of guinea pigs in this study. Other monoclonal antibodies were non-reactive with the isolate.

6.3.3 Clinical observations in guinea pigs experimentally-infected with *Leptospira interrogans* serovar *icterohaemorrhagiae*

Following 12-hours post-infection (p.i), some of the guinea pigs showed dullness, anorexia and dehydration (12/20). At day 1 p.i., dullness, anorexia and dehydration persisted, and in addition to this most of these animals had starry hair coat and pale mucous membrane.

At day 2 p.i., 11 guinea pigs were slightly jaundiced, while two of the guinea pigs showed petechial hemorrhages and mucopurulent discharges on the conjunctiva. At day 3 p.i., all the above signs persisted with prostration. At day 4 p.i., same clinical signs were observed but with much severity and presence of muco-catarrhal exudate mixed with partially formed feaces (Fig. 6.2). At day 5 p.i., the clinical signs persisted, with lethargy.

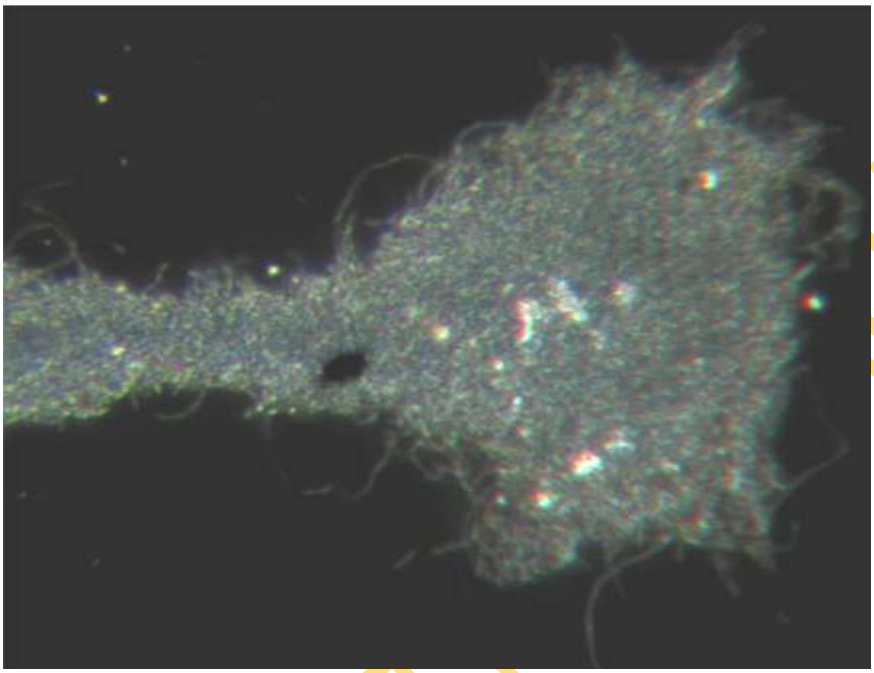

Fig.6.1 Photomicrograph of the *Leptospira interrogans* serovar *icterohaemorrhagiae* colony viewed under dark- field microscopy with the characteristic tread-like morphology. x 750.

6.3.4 Isolation of leptospiral organism from different organs in infected guinea pigs.

Table 6.1 below represent cultural isolation of leptospiral organism from different organs at different times (12hrs, days 1, 3, 5 and 7 p.i.). Leptospires were recovered from all the organs examined at different times from 12 hrs until day 7 p.i. The study showed that at 12 hrs and day 1, the organism was detected with EMJH medium from the liver, heart, lung, pancreas, adrenal gland, kidney, spleen and brain, but was not detected in the heart at 12hrs p.i.

At day 3 p.i, the organism was detected only in the liver, lung, kidney and pancreas. This coincided with the presence of haemorrhages at day 3 p.i in the lung.

At day 5 p.i, it was only detected in the liver, kidney and lung, but at day 7 p.i. the leptospiral organism was present only in the kidney and absent in the remaining organs.

Fig.6.2 Photomicrograph of 2 of the infected guinea pigs with one showing postration with soft mucoid feaces and the other showing curdling during one of the experimental phase.

Table 6.1 Cultural isolation of leptospiral organism at different days from different organs of guinea pigs using EMJH medium

Time	liver	kidney	Heart	Brain	Lung	Spleen	Pancreas	Adrenal gld
½ day	+	+	-	+	+	+	+	+
Day 1	+	+	+	+	+	+	+	+
Day 3	+	+	-	-	+	-	+	-
Day 5	+	+	-	-	+	-	-	-
Day 7	-	+	-	-	-	-	-	-

- = *negative*, + = *positive*

6.3.5 Detection of leptospiral DNA in organs of infected guinea pigs using PCR

Figure 6.3. depicts the gene amplicons of 285bp, amplified from different organs such as brain, heart, adrenal gland, spleen. Although leptospiral pathogenic genes were amplified in some organs, but there were variations in the intensity of the amplicons. With the forward and reverse primers used in this study (G1/G2), PCR amplified the leptospiral DNA from different organs at different times. At 12hrs, leptospiral DNA was amplified from the brain, pancreas, heart and the adrenal gland. On day 3 p.i., leptospiral DNA amplifications were only observed in pancreas and the adrenal gland, but amplification of leptospiral DNA in the brain and heart was not observed. From day 5 p.i. all the organs of animals sacrificed were found to be negative by the PCR except kidney.

6.3.6 Dissemination kinectics of *Leptospira icterohaemorrhagiae* into different organs in infected guinea pigs model using immunohistochemistry

Figure 6.4 below shows dissemination kinectics of *Leptospira icterohaemorrhagiae* into different organs in infected guinea pigs model using slides stained immunohistochemically. In this study, kidney had the highest leptospire load throughout the period of this study with the highest peak at day 7. The peak leptospire load in the liver and lung was day 5 p.i. (9.5±0.35 and 3.0±0.35 respectively), but in the spleen, and the brain it was day 3 p.i. and day 1 p.i. respectively (5.0±0.65 and 3.0±0.65 respectively). The spleen showed the highest load of leptospire at day 3 p.i compared with other organs. Leptospire were not observed in the brain beyond day 1 p.i., but there was steady increase in the leptospire load in the kidney and the liver from day 1 p.i. to day 7 p.i., except for liver in which there was decrease in the leptospiral load at day 7 p.i.

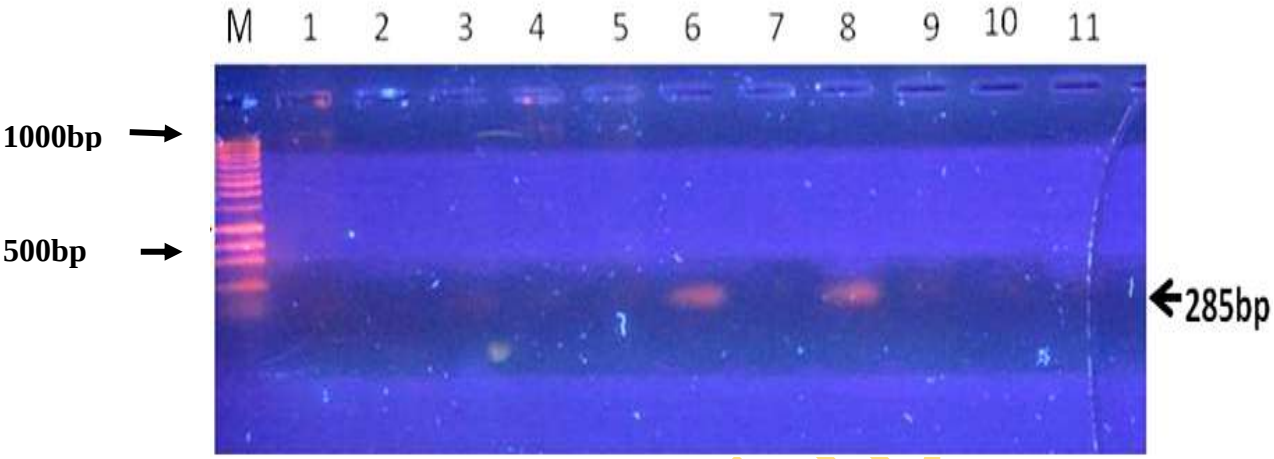

Fig. 6.3 Polymerase chain reaction of the tissues of guinea pig infected with *Leptospira interrogans* serovar *icterohaemorrhagiae*. (Lane M = DNA Marker 1kb , Lane **1** = kidney at day 3 p.i., Lane **2**= Brain at 12 hrs p.i., Lane **3**= Pancreas at 12hrs p.i., Lane **4** = Spleen at day 3 p.i., Lane **5** = Heart at 12hrs, Lane **6** = Brain at 12 hrs p.i., Lane **7** = Spleen at 12 hrs p. i., Lane **8** = Adrenal gland at day 3 p.i., Lane **9** = Pancreas at day 3 p.i., Lane **10** = Brain at day 3 p.i., Lane **11** = Adrenal gland at 12 hrs p.i.

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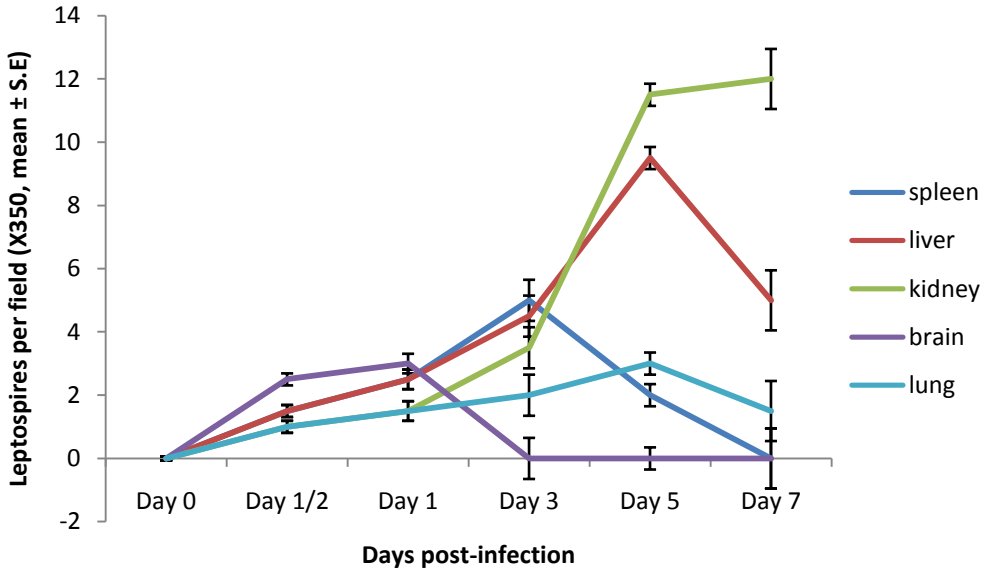

Fig. 6.4 Graph showing the distribution of *leptospira icterohaemorrhagiae* at different time interval in organs such as spleen, liver, kidney, brain and the lung in infected guinea pigs. (Results were expressed as mean± standard error).

6.3 .7 Haematological findings in guinea pigs experimentally-infected with *Leptospira interrogans* serovar *icterohaemorrhagiae.*

The hematological results of this study are depicted in Figures.6.5- 9and appendixies 2-6. At 12 hr. p.i., all the hematological parameters (Hb, MCHC, MCV, PCV, and RBC) were not significantly different in the infected group (13.27±0.81g/dl, 38.63±1.45g/dl, 94.20±7.42fl, 34.33±1.33%, and 3.67±0.15x$10^6$$mm^3$ respectively) compared with the control group (16.30±0.20g/dl, 39.35±1.95g/dl, 99.30±15fl, 41.50±1.50% and 4.30±0.80 x$10^6$$mm^3$ respectively), however, there was slight to moderate decrease in the mean values of PCV at this period.

At day 1 p.i., all the hematological parameters were not also significant, except the mean values of the MCHC which were observed to be significantly higher in the infected guinea pigs (38.23±0.93g/dl) compared with the control group (33.60±0g/dl).

At days 2 and 3 p.i., most of the parameters were also not significantly different, except moderate decrease in the mean values of PCV and MCV in the infected guinea pigs compared with the control group at these periods.

At day 4 p.i, the mean values of RBC in the infected guinea pigs (3.90±0.17 x$10^6$$mm^3$) were observed to be significantly lower ($P<0.05$) than that of the control group (5.00±0.20 x$10^6$$mm^3$).

At day 5 p.i., most of the parameters were not significantly different but the mean values of PCV and Hb of the infected guinea pigs (28.33±2.19 and 7.97±1.68 respectively) were observed to be significantly lower ($P<0.05$) than that of the control group (39.00±1.00 and 13.95±0.15g/dl respectively).

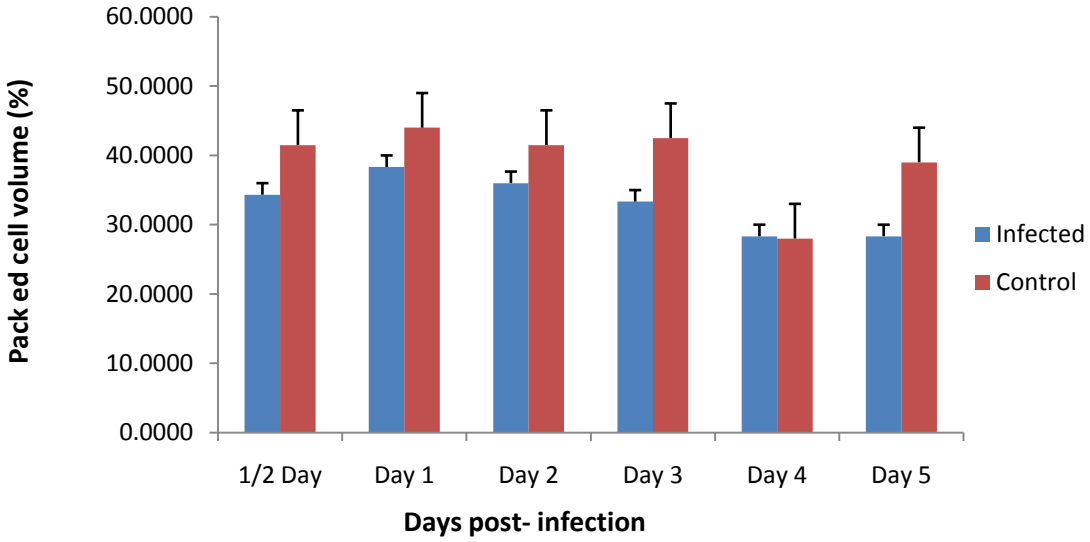

Fig. 6.5 Bar-chart showing the mean (±SEM) values of PCV of leptospiral-infected guinea pigs at different days p.i. compared with the control groups.

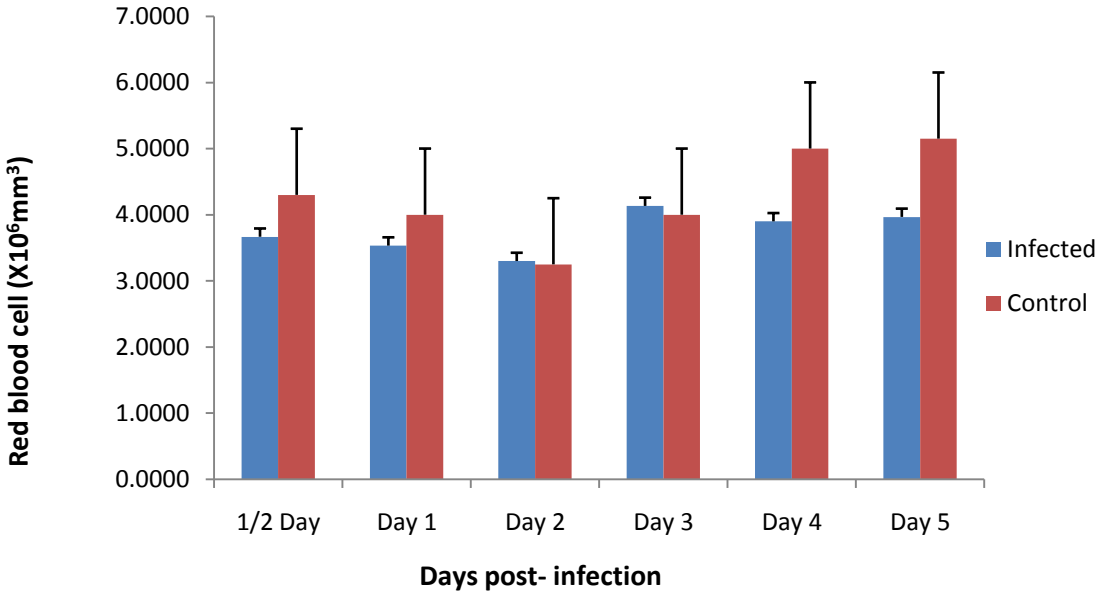

Fig. 6.6 Bar-chart showing the mean (±SEM) values of RBC of leptospiral-infected guinea pigs at different days p.i. compared with the control groups.

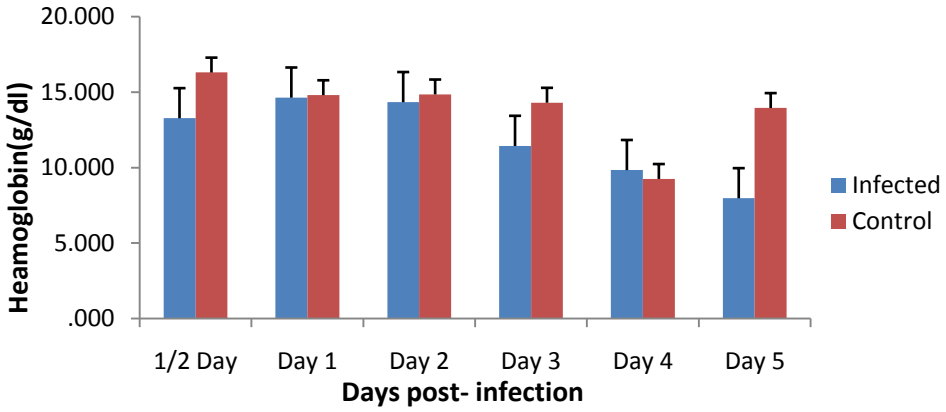

Fig. 6.7 Bar-chart showing the mean (±SEM) values of Hb in leptospiral-infected guinea pigs at different day's p.i. compared with the control groups.

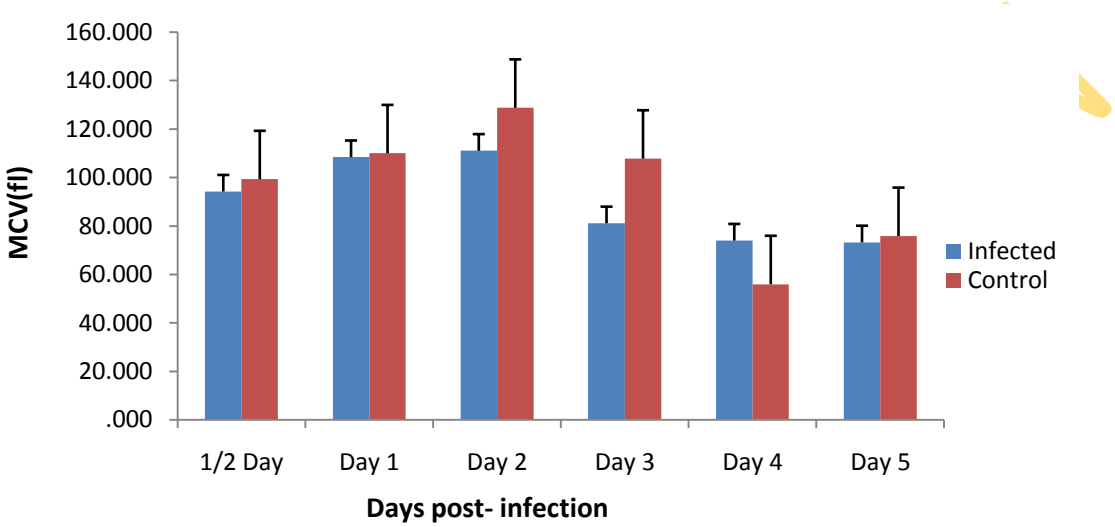

Fig. 6.8 Bar-chart showing the mean (±SEM) values of MCV in leptospiral-infected guinea pigs at different day's p.i. compared with the control groups.

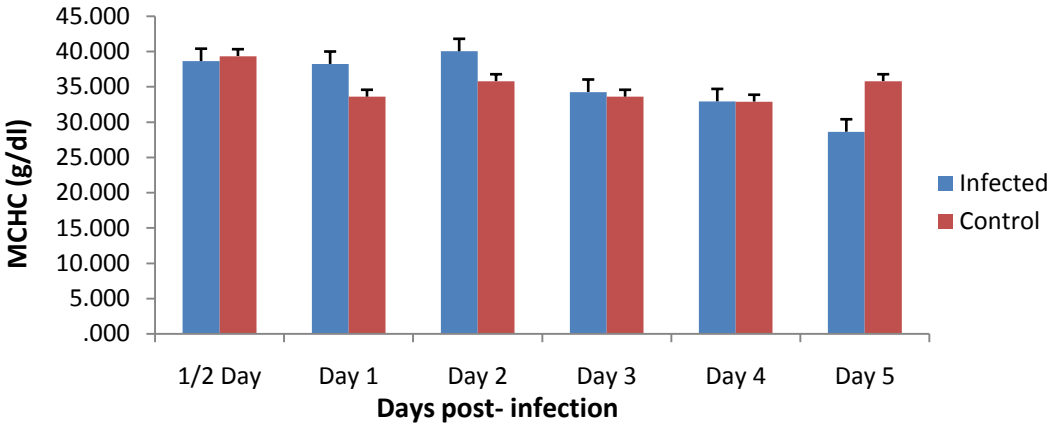

Fig. 6.9 Bar-chart showing the mean (±SEM) values of MCHC in leptospiral-infected guinea pigs at different day's p.i. compared with the control groups.

6.3.8 Reticulocyte response in infected and control guinea pigs

At 12 hr. p.i., reticulocytes were not detected in the infected guinea pigs compared with the control group , but at days 1 and 2 p.i., the mean values of the reticulocyte count observed in the infected guinea pigs ($7.33\pm4.62x10^6mm^3$ and $7.87\pm4.50x10^6mm^3$ respectively) were significantly higher than that of the control group ($3.00\pm1.00x10^6mm^3$ and 5.50 ± 2.00 $x10^6mm^3$ respectively).

At days 3, 4, and 5 p.i., the mean values of the reticulocyte count of infected guinea pigs (7.17 ± 4.16 $x10^6mm^3$, 3.80 ± 0.95 $x10^6mm^3$, and 2.07 ± 1.35 $x10^6mm^3$ respectively) were significantly decreased ($P<0.05$) compared with the control group (17.80 ± 16.00 $x10^6mm^3$, 12.40 ± 2.00 $x10^6mm^3$, and 2.60 ± 0.10 $x10^6mm^3$ respectively) (Fig.6.10 and appendix 7)

The reticulocyte count was positively correlated with the PCV ($R^2=0.273$) but not significant ($P>0.05$) in the infected guinea- pigs as shown in the Figure 6.11 below.

For WBC, there was mild leukocytosis at 12hrs p.i. compared with the control. But day 1, 3, and 5, significant leucopenia was observed, compared with the control (Fig.6.12). Thrombocytopenia was observed throughout the period of this study compared with the control (Fig.6.13)

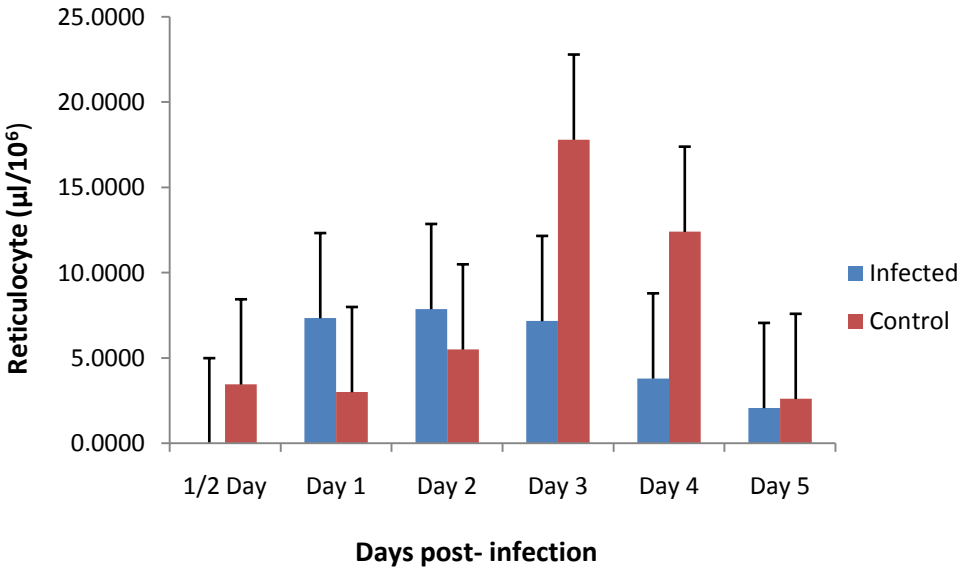

Fig. 6.10 Bar-chart showing the mean (±SEM) values of reticulocyte counts of *Leptospira*-infected guinea pigs at different days p.i. compared with the control groups.

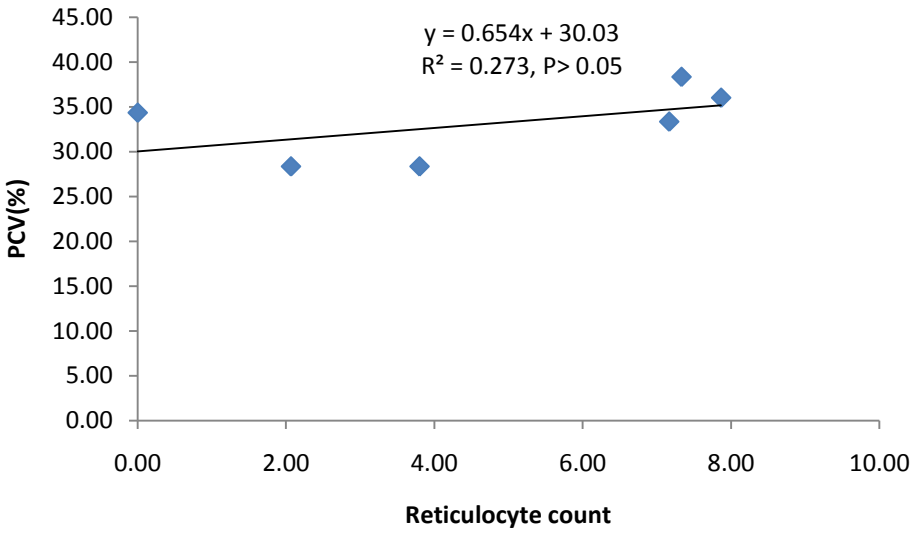

Fig. 6.11 Graph of linear regression of PCV and reticulocyte count showing positively correlated but not significant change.

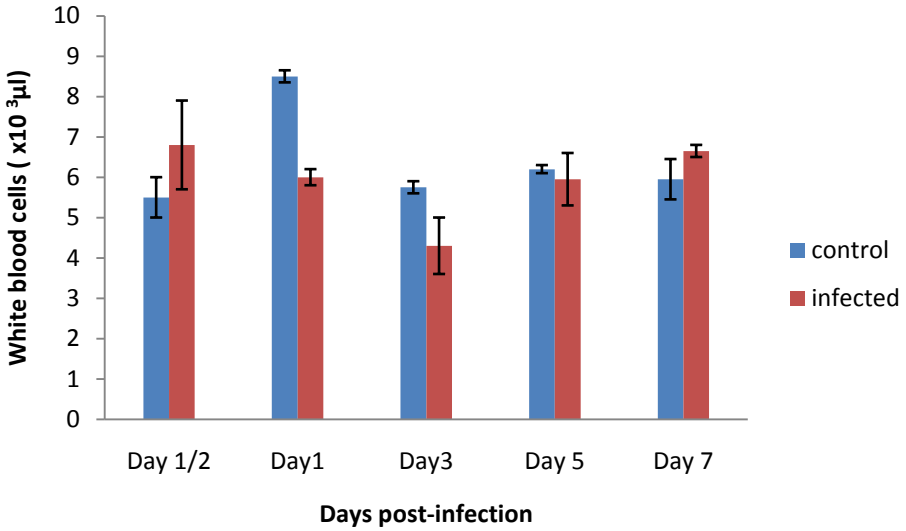

Fig. 6.12 Bar-charts showing the mean (±SEM) values of WBC of leptospiral-infected guinea pigs at different day's p.i. compared with the control group.

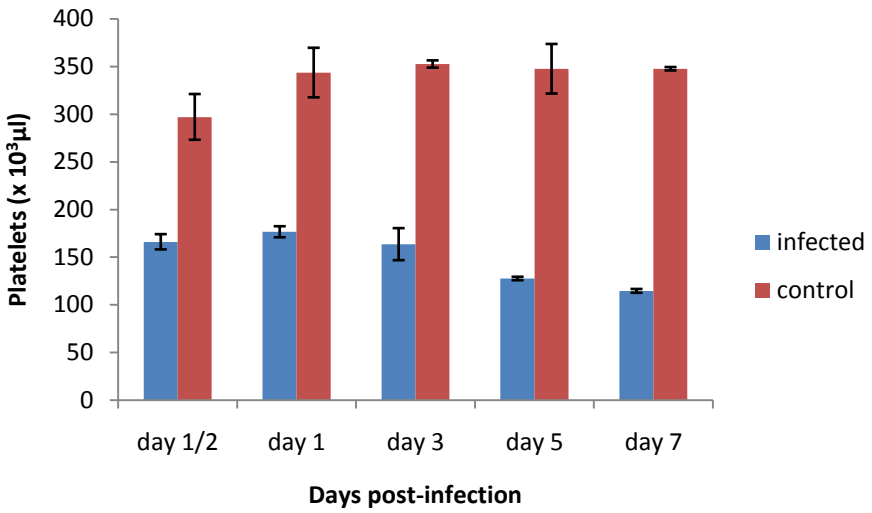

Fig. 6.13 Bar-charts showing the mean (±SEM) values of platelets of leptospiral-infected guinea pigs at different day's p.i. compared with the control group.

6.3.9 In vivo lipid peroxidation in infected and control guinea pigs

The plasma thiobarbituric acid reactive substances (TBARS) concentration, indicated by malondialdehyde (MDA) concentration was observed to be higher in the infected guinea-pigs at days 2 and 3 p.i., (1.95±1.79 $x10^6$µm/ml, and 0.45±0.08 $x10^6$µm/ml respectively) than in the control group (0.38±0.34 $x10^6$µm/ml, and 0.22±0.17 $x10^6$µm/ml respectively). Although there was no significant change between PCV and in vivo MDA, but they were positively correlated ($P>0.05$) ($R^2=0.070$) as shown in the Figure 6. 14-15 below.

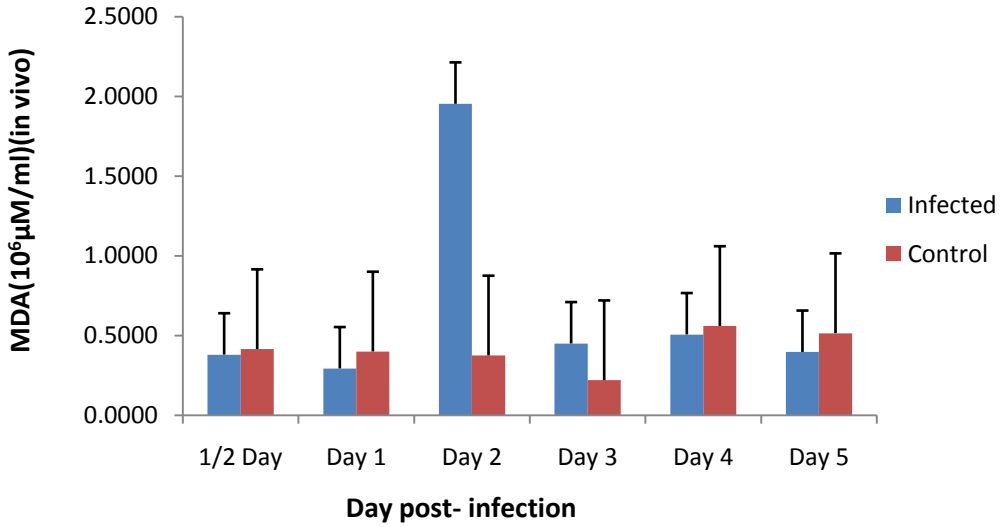

Fig.6.14 Bar-chart showing the mean (±SEM) values of in vivo MDA of leptospiral-infected guinea pigs at different hours p.i. compared with the control groups.

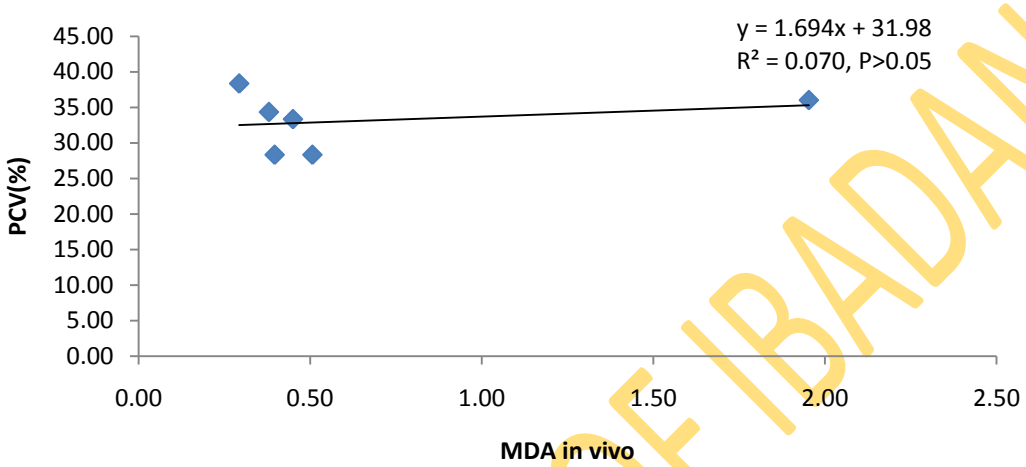

Fig.6.15 Graph of linear regression with positive correlation between packed cell volume and in vivo MDA.

6.3.10 In vitro peroxidation in infected and control guinea pigs.

Generally, the in vitro lipid peroxidation mean values were significantly higher ($P<0.05$) in the infected guinea pigs than in the control group, especially at day 3 p.i. as shown in the Figure6.16 below. PCV was negatively correlated with in vitro lipid peroxidation ($R^2 =0.089$) in the infected guinea pigs as shown in the figures 6. 17 below.

In addition, in vivo MDA was positively correlated ($P<0.05$) with in vitro lipid peroxidation ($R^2 =0.016$) in the infected guinea pigs but there was no significant difference ($P>0.05$) as shown in the Figure 6. 18 below

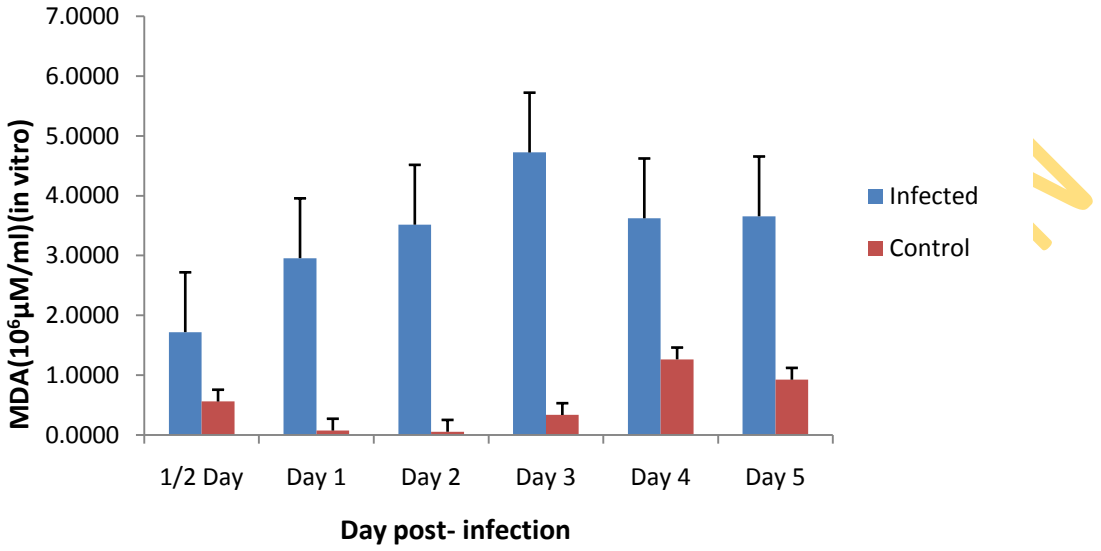

Fig.6.16 Bar-chart showing the mean (±SEM) values of in vitro lipid peroxidation of leptospiral-infected guinea pigs at different days p.i. compared with the control groups.

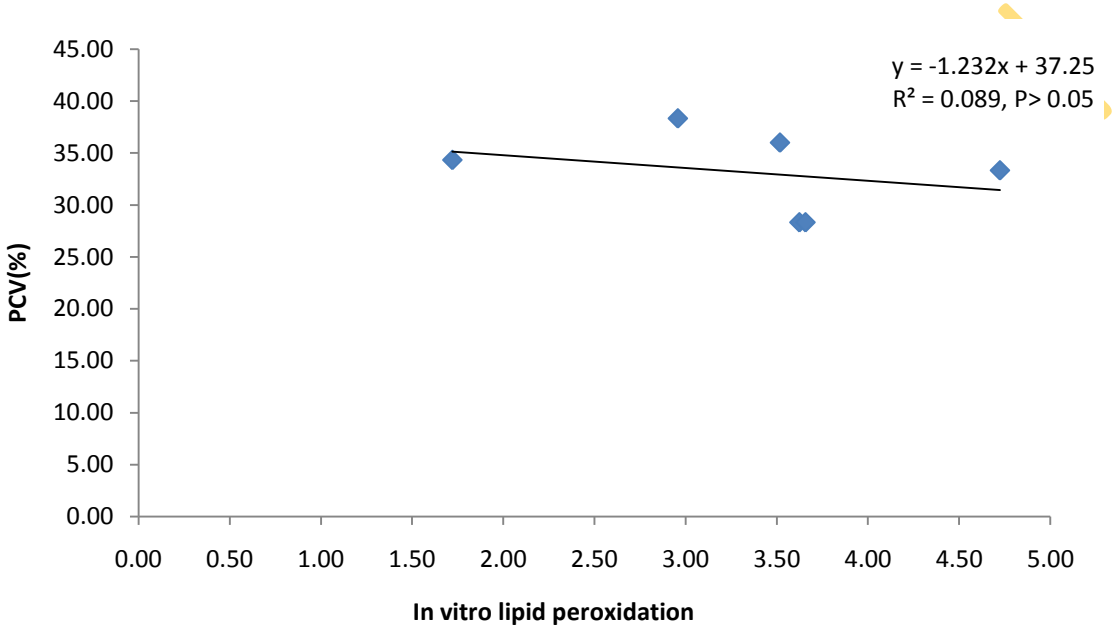

Fig.6. 17 Graph of linear regression with no significant negative correlation between packed cell volume and in vitro lipid peroxidation.

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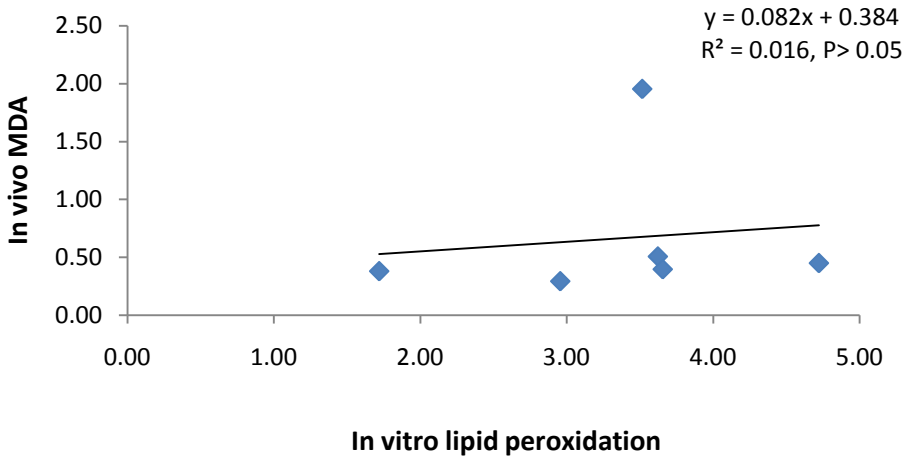

Fig.6.18 Graph of linear regression showing positive correlation between in vivo MDA with in vitro lipid peroxidation.

6.4. Serum biochemical parameters in *Leptospira icterohaemorrhagiae* infected and control guinea pigs

There was no significant change in the mean values of the total protein throughtout the period of this study except at day 7 p.i. in which mild significant decrease was observed**,** compared with the control. No significant change was present in the mean values of albumin and globulin in this study (appendix).

There was significant increase ($P<0.05$) in the mean values of ALT at day 3, 5 and 7 p.i. (61.00±0.40µ/L, 67.07±0.86µ/L and 69.25±0.25µ/L respectively) in the infected groups compared with the control groups (43.15±0.85 µ/L, 49.50 ± 0.80 µ/L, 49.95 ± 0.75 µ/L and 43.70±5.00 µ/L respectively) (Fig.6.19)

Although, the mean values of ALP of the infected groups were slightly higher than the control groups at days 1, 3, 5 and 7 p. i, there was no significant difference throughtout the period of this study.

Considerable significant increase ($P<0.05$) was observed in the urea levels of infected guinea pigs, especially at 12hrs and day 1 p.i. compared with the control groups. At day 3-7 p.i., there was no significant difference between the infected and the control groups, but there were slight to moderate increase in the mean values of urea of infected guinea pigs at this period (Fig.6.20.)

Despite the fact that there were slight to moderate increase in the mean values of creatinine in infected guinea pigs at day 7 p.i., there was no significant increase in the mean values of infected guinea pigs at 12hrs, 1, 3 and 7 compared with the control groups. But, slight significantly increase was observed at day 5 p.i. compared with the control (Fig.6.21) .

Although, no significant change was observed in the mean values of potassium at 12hrs p.i., but at day 1 p.i., there was slight significant increase ($P<0.05$) in the mean value of potassium

compared with the control group. At day 5-7 p.i., there was significant decrease in the mean values of potassium in the infected group compared with the control (Fig.6.22).

Throughout the period of the experiment, there was no significant change in the mean values of the bicarbonate and chloride, except at days 7 and 5 p.i. (respectively), in which there was significant change in the bicarbonate (control=22.05±0.05mmol/dl; infected= 26.05±0.05 mmol/dl) and the chloride (control = 92.30±0.90 mmol/dl, infected = 109.57±3.74 mmol/dl) values.

The mean values of bilirubin were not significantly different throughout the period of this study, but there was moderate increase in the mean values of bilirubin in the infected guinea pigs from day 1 to 7 p.i. (Fig.6.23).

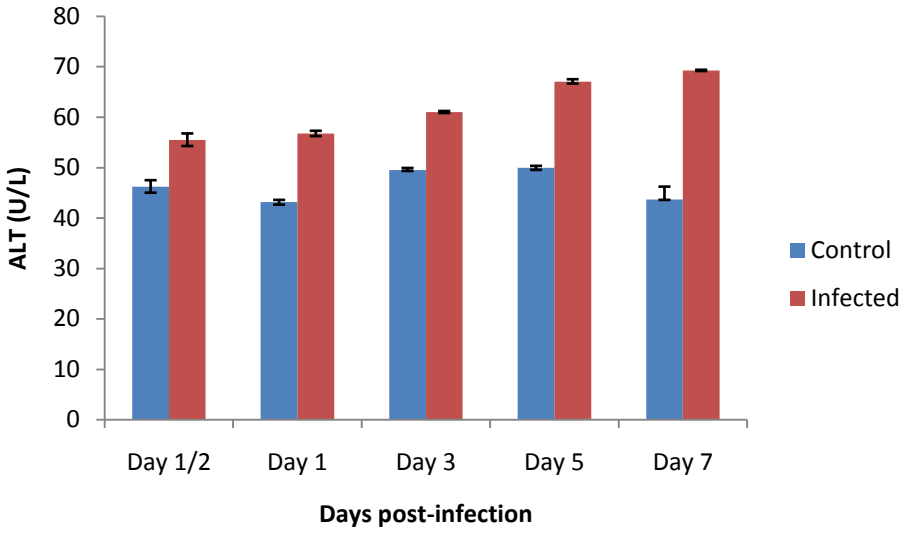

Fig.6.19. Bar-chart showing the mean (±SEM) values of ALT of leptospiral-infected guinea pigs at different days p.i. compared with the control groups.

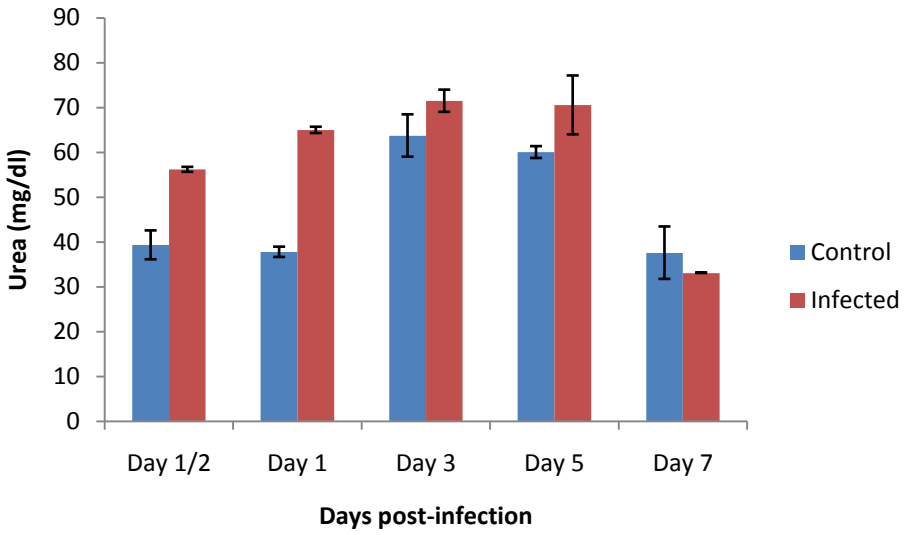

Fig.6.20 Bar-chart showing the mean (±SEM) values of urea of leptospiral-infected guinea pigs at different days p.i. compared with the control groups.

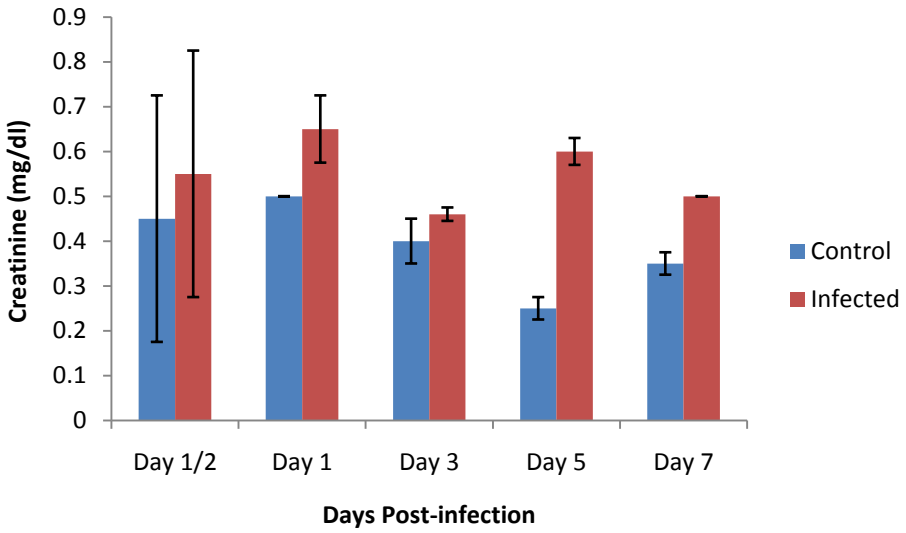

Fig.6.21 Bar-chart showing the mean (±SEM) values of creatinine of leptospiral-infected guinea pigs at different day's p.i. compared with the control groups.

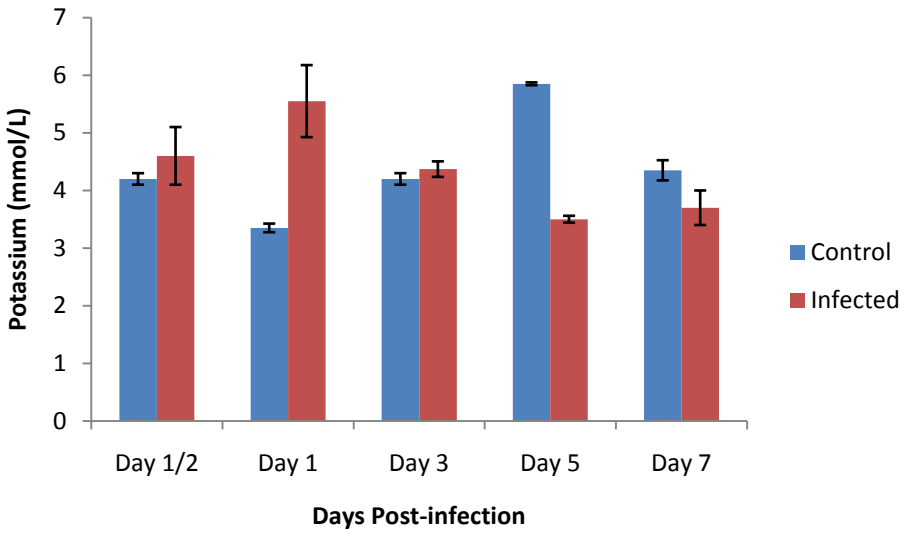

Fig.6.22 Bar-chart showing the mean (±SEM) values of potassium of leptospiral-infected guinea pigs at different days p.i. compared with the control groups.

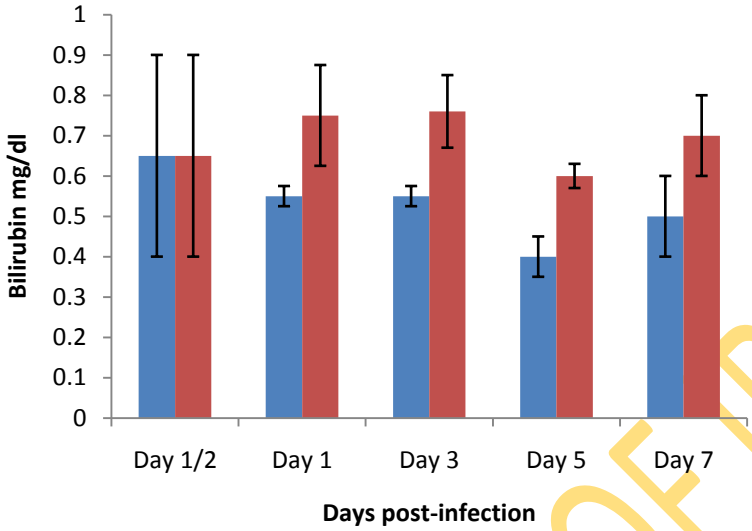

Fig.6.23 Bar-chart showing the mean (±SEM) values of bilirubin of leptospiral-infected guinea pigs at different days p.i. compared with the control groups.

6.5 Gross pathological observations in guinea pigs experimentally-infected with L*eptospira interrogans* serovar *icterohaemorrhagiae*

The gross morphological alterations in organs of guinea pigs experimentally-infected with *leptospira interrogan* serovar *icterohaemorrhagiae* are represented in Table 6.2 and 6.2.1.

All the infected animals were slightly dehydrated and emaciated at day 1 p.i. The lungs were not collapsed but were slightly congested and oedematous (4/4) at day 1. There was slight subcutaneous jaundice (3/4) at day 3 p.i. Multiple foci of petechial and ecchymotic haemorrhages were observed on the pleura surfaces of the lungs and the thoracic wall (2/4) at days 3 and 5 p.i.

Suffusion haemorrhages was observed on the gastric mucosa (Figs.6.24-25a&b) with segmental petechiations and ecchymoses along the small and the large intestines. The liver was slightly enlarged and congested (3/4). Throughout the duration of the study, there were variations in the size and gross appearance of the spleen, at 12 hrs, and days 1 and 3 some showed splenomegaly while at days 5 and 7 some presented mild to moderate splenic atrophy. The interlobular septae of the pancreas were prominent with oedema fluid and multiple foci of petechiations were also observed at day 3 p.i. The meningeal blood vessels were markedly injected at days 1 (2/4) and 3 p.i. (1/4).

Table 6.2. Semi-quantification of gross morphological alterations in organs of guinea pigs experimentally-infected with *Leptospira interrogans* serovar *icterohaemorrhagiae.*

Lesions	**control (n)**	½ Day	**Day 1**	**Day 3**	**Day 5**	**Day 7**
Dehydration and emaciation	-	+	++	+	++	+
Subcutaneous jaundice	-	-	+	++	++	+
Petechiations &Echymoses	-	-	++	++	+	+
Haemorrhagic gastroenteritis	-	-	++	+++	+	+
Splenomegaly	-	+	++	++	-	-
Splenic atrophy	-	-	-	-	+	++
Hepatomegaly and jaundice	-	+	++	++	+	+
Pulmonary congestion and oedema	-	++	+++	+++	++	++
Pulmonary haemorrhages	-	-	+	++	+++	+
Meningeal congestion	-	++	++	+	-	-
Pancreatic haemorrhages	-	-	-	+	-	-

- = Absent, + = mild, ++ = moderate, +++ = marked or severe.

Table 6.2.1 Gross morphological alterations in organs and the number of guinea pigs involved in *Leptospira interrogans* serovar *icterohaemorrhagiae infection.*

Lesions	Contl (10*)	½ Day (4)	Day 1 (4)	Day 3 (4)	Day 5 (3)	Day 7 (3)	Total (18)	(%)	95%CI Lower Upper
Dehydration and emaciation	0	3	2	1	1	1	8	(44.4)	21.0 – 67.0
Subcutaneous jaundice	0	0	1	2	2	0	5	(27.8)	07.0 - 49.0
Petechiations and echymoses	0	0	3	3	3	2	11	(61.1)	38.0 - 84.0
Haemorrhagic gastroenteritis	0	0	2	3	2	1	8	(44.4)	21.0 – 67.0
Splenomegaly	0	1	3	3	0	0	7	(38.9)	16.0 - 62.0
Splenic atrophy	0	0	0	1	2	2	5	(27.8)	07.0 - 49.0
Hepatomegaly and jaundice	0	1	3	3	2	1	10	(55.6)	33.0 - 59.0
Pulmonary congestion and oedema	0	3	4	3	3	3	16	(88.9)	74.0 - 100
Pulmonary haemorrhages	0	0	1	3	3	2	9	(50.0)	27.0 - 73.0
Meningeal congestion	0	3	2	1	0	0	6	(33.3)	11.0 - 55.0
Pancreatic haemorrhages	0	0	0	1	3	0	3	(22.2)	03 - 41.0

** =Ten animals were used throughout the study in the control group i.e two guinea pigs at each period.*

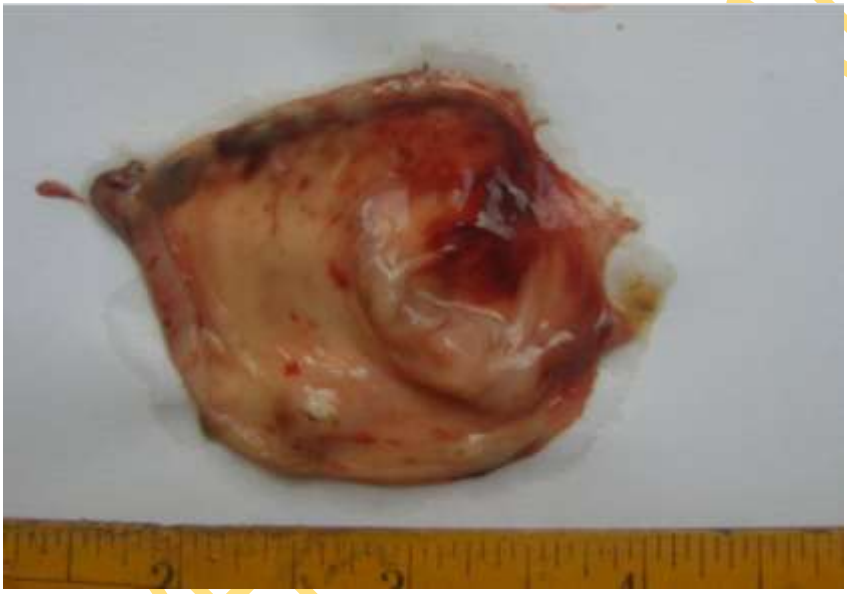

Fig.6.24 Gross appearance of the gastric mucosa showing petechial and suffusion haemorrhages at day 1 p.i.

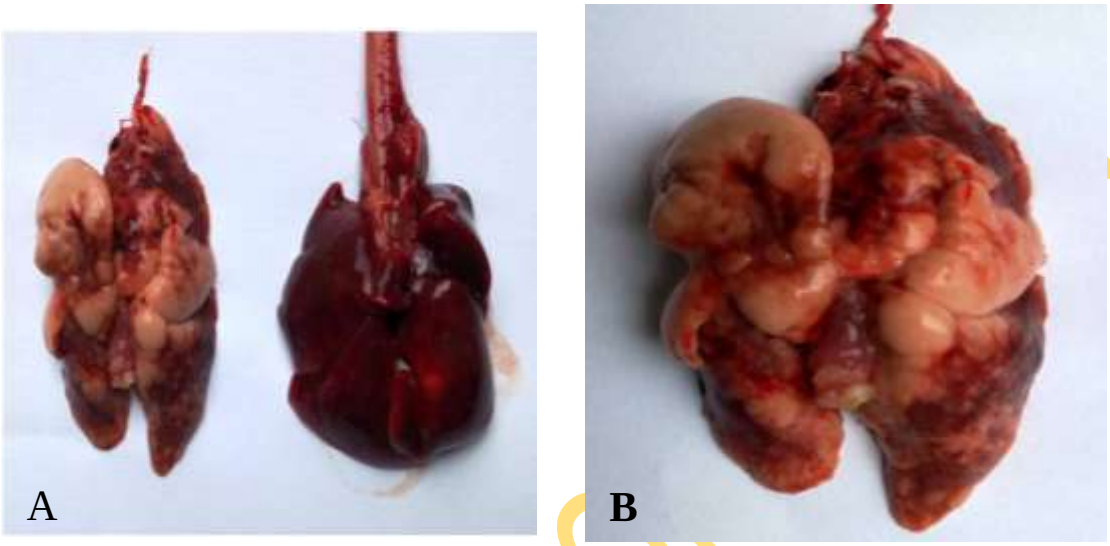

Fig.6.25a & b Gross appearance of the lungs showing non-collapsed, congested and haemorrhagic lungs with severe emphysema at day 3 p.i. (A&B).

6.6.0 Histopathological changes in different organs of guinea pigs experimentally-infected with *leptospira icterohaemorrhagiae*

Several histopathological changes were observed in the liver, heart, pancreas, brain, adrenal gland, kidney, lungs, and spleen. The most prominent lesions were observed in guinea pigs killed at day 3, 5 and 7 p.i.

6.6.1 Hepatic histopathological changes in guinea pigs infected with *leptospira icterohaemorrhagiae*

Table 6.3.1-2 above and Figs. 6.26-27 depicts hepatic histopathological changes with the severity of the lesions in guinea pigs infected with *leptospira icterohaemorrhagiae.* Histopathological changes such as mild to moderate portal and sinusoidal congestion (16/18, 88.9%, 95%CI 73 – 100%) Kupffer cells hyperplasia (13/18, 72.2%, 95%CI 51 -93%), mild vacuolar degeneration (10/18, 55.6%, 95%CI 33 -59%), mild erythrophagocytosis and moderate periportal oedema (12/18, 66.7%, 95%CI 45 -89%) were the early lesions in the livers of guinea pigs at 12 hrs and day 1 p.i. At day 3 to 7 p.i., there was increase in severity of the earlier changes with focal areas of hepatic necrosis (11/18, 61.1%, 95%CI 38 -84%) with some sections showing vacuolar degeneration, moderate to marked periportal oedema and congestion. There was mild to moderate loss of cohension and disorganization of hepatic cords (6/18, 33.3%, 95%CI 11-55%) with evidence of regeneration such as binucleation and cytomegaly. Immunohistochemically, leptospire fragments and granules staining reddish were observed in the sinusoids and within the cytoplasm of Kupffer cells at days 3 and 5 p.i. (Figs 6.28a, b & c)

Table 6.3.1. Semi-quantification of hepatic histopathological alterations in guinea pigs infected intraperitoneally with *Leptospira interrogans* serovar *icterohaemorrhagiae.*

Lesions	**Control (10)**	**½ day**	**day 1**	**day 3**	**day 5**	**day 7**
Portal and sinusoidal congestion	+	+	++	++	++	++
Periportal oedema	-	++	++	++	+++	+++
Sinusoidal dilatation	-	+	+	+	+	+
Hepatic necrosis	-	-	+	++	++	++
Dissociation and disorganization	-	-	+	+	+	+
Kupffer cell hyperplasia	-	+	++	++	+	++
Erythrophagocytosis	-	-	+	+	++	++
Haemosiderosis	-	+	++	++	++	++
Vacuolar degeneration	+	+	+	++	++	+++
Binucleation	+	-	-	+	+	++
Cytomegaly	-	-	-	+	+	+

- = Absent, + = mild, ++ = moderate, +++ = marked or severe.

Table 6.3.2 The number of animals involved and the hepatic histopathological alterations in guinea pigs infected intraperitoneally with *Leptospira interrogans* serovar *icterohaemorrhagiae*. Data on the numbers of guinea pigs involved were also presented.

								95%CI	
Lesions /(No of animals killed)	Control **(10*)**	**½ Day (4)**	**Day 1 (4)**	**Day 3 (4)**	**Day 5 (3)**	**Day 7 (3)**	**Total (18)**	**(%)**	**Lower Upper**
Portal & sinusoidal congestion	0	4	4	3	3	2	16	88.9	73.0 - 100
Periportal oedema	0	2	4	2	2	1	11	61.1	38.0 - 84.0
Sinusoidal dilatation	0	1	1	2	1	2	7	38.9	16.0 - 62.0
Hepatic necrosis	0	0	2	3	3	3	11	61.1	38.0 - 84.0
Dissociation and disorganization	0	0	0	2	2	2	6	33.3	11.0 - 55.0
Kupffer cell hyperplasia	0	2	2	3	3	3	13	72.2	51.0 - 93.0
Erythrophagocytosis	0	0	3	4	2	3	12	66.7	45.0 - 89.0
Haemosiderosis	0	0	1	1	3	2	7	38.9	16.0 - 62.0
Vacuolar degeneration	4	1	1	3	3	2	10	55.6	33.0 - 59.0
Binucleation	2	0	0	2	2	1	5	27.8	07 - 49.0
Cytomegaly	3	2	1	2	2	2	9	50.0	27.0 - 73.0

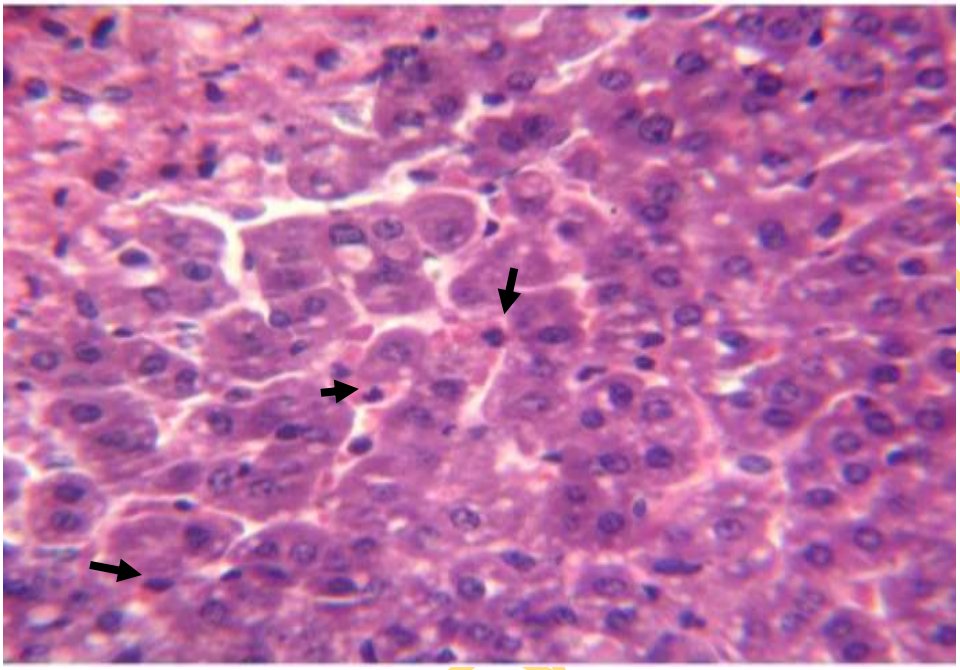

Fig. 6.26 Photomicrograph of the liver section showing mild loss of hepatic cords, mild hepatic vacuolations and erythrophagocytosis by the Kupffer cells at day 5 p.i. (arrows) H & E. x 350.

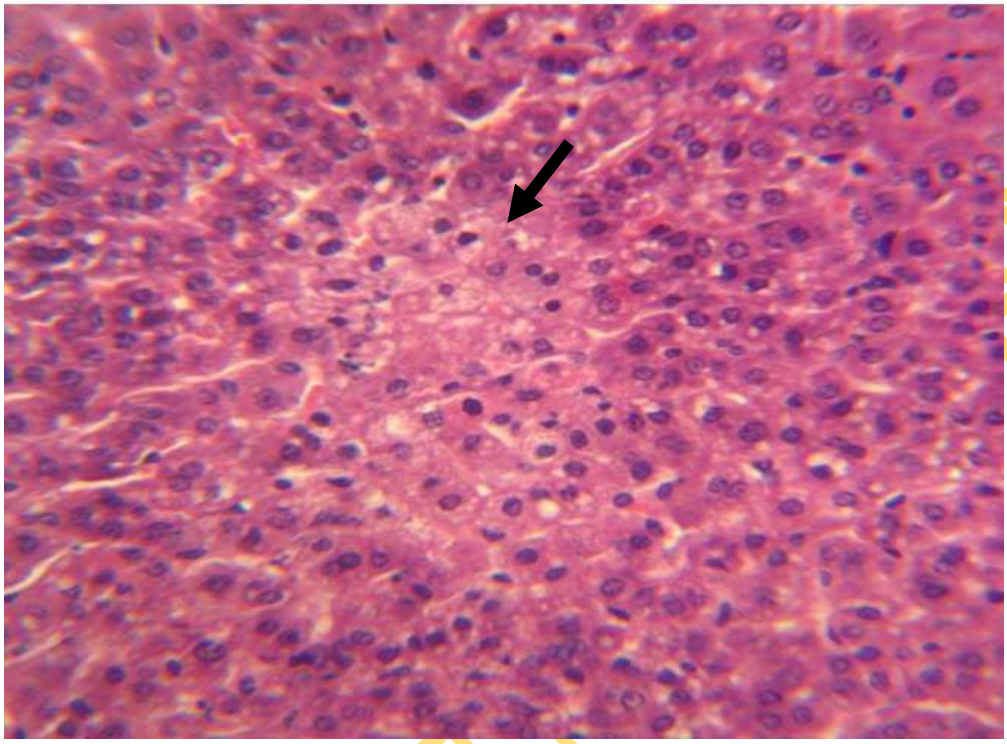

Fig. 6.27 Photomicrograph of the liver section showing hepatic degeneration and necrosis (arrow) at day 5 p.i. x 350 H & E.

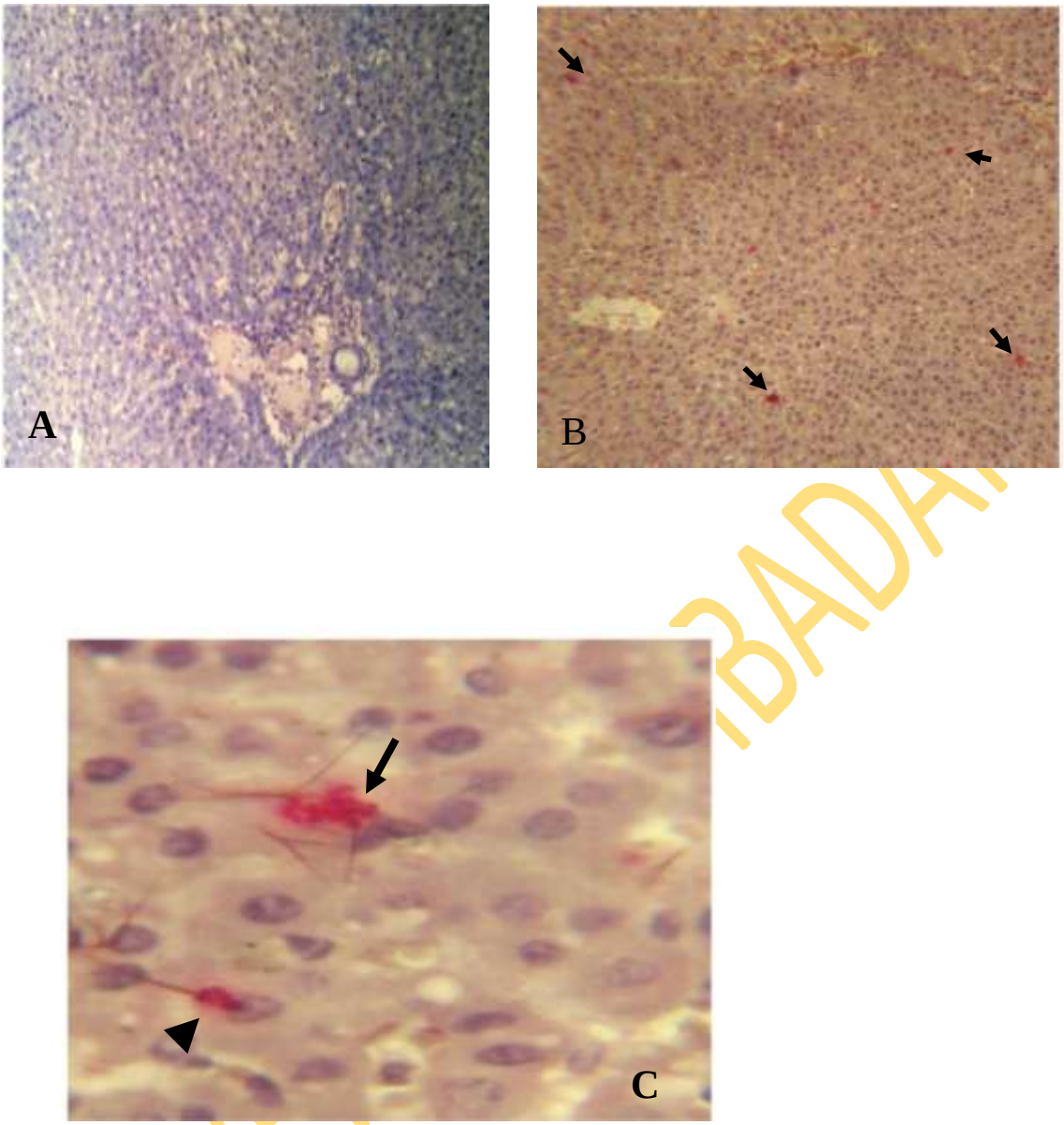

Fig. 6.28 a, b & c. Photomicrograph of (A) normal and (B) immunohistochemical detection of *Leptospira interrogans* serovar *icterohaemorrhagiae* in guinea pig liver. Leptospiral debris and fragment stained reddish in the cytoplasm of hepatocytes (arrow head), and the sinusoids (arrow) at day 3 p.i x 200.(C) Higher magnification of B. Alkaline phosphatase-fast red counterstained with heamatoxylin x 450.

6.6.2 Pulmonary histopathological changes in guinea pigs infected with *leptospira icterohaemorrhagiae*

Table 6.3.3-4 and figures 6.29-31a & b depicts pulmonary histopathological changes and its severity in guinea pigs infected intraperitoneally with *Leptospira icterohaemorrhagiae*. The pulmonary vessels were markedly congested with red blood cells admixed with protenacious materials in 13 (72.2%, 95%CI 51 – 93%) out of 18 animals. Multifocal to locally diffuse areas of haemorrhages were present in the pulmonary parenchyma in 9 (50%, 95%CI 27 - 73%), the pulmonary blood vessels were markedly congested and alveolar spaces contained multifocal to locally extensive, deeply oesinophilic proteinaceous oedema fluid. The haemorrhagic lesion was more severe and the alveolar spaces were filled with erythrocytes and alveolar macrophages with moderate heamosiderosis in 7 (38.9%, 95%CI 16 -62%). There was marked interstitial pneumonia in 7 animals (38.9%, 95%CI 16 -62%), characterized by thickened alveolar wall which consisted of congested vessels, erythrocytes, oedema fluid, alveolar macrophages, lymphocytes and few neutrophils. There was marked erythrophagocytosis and haemosiderosis in the alveolar spaces and interstitium. The pulmonary arterial and capillaries vessels contained varying sized thrombi in the lumina with some attached to the vascular mucosa with thin fibrin strands and some totally obliterating the lumina of the vessels in 6 (33.3%, 95%CI 11 -55%) guinea pigs. Immunohistochemical staining revealed leptospires granules within the cytoplasm of the alveolar macrophages in the alveolar septae at days 3 and day 7 p.i. Leptospire in the lung were less in number compared to those in the liver and the kidney.

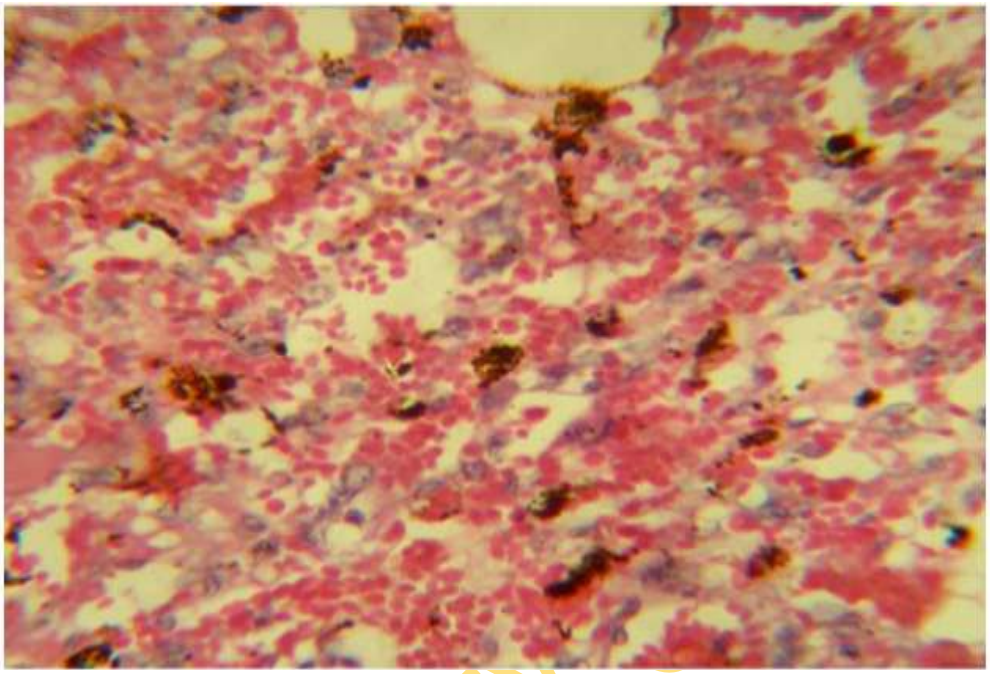

Fig. 6.29 Photomicrograph of the lung sections at day 3 p.i. showing severe haemorrhages with haemosiderosis. H & E x 350

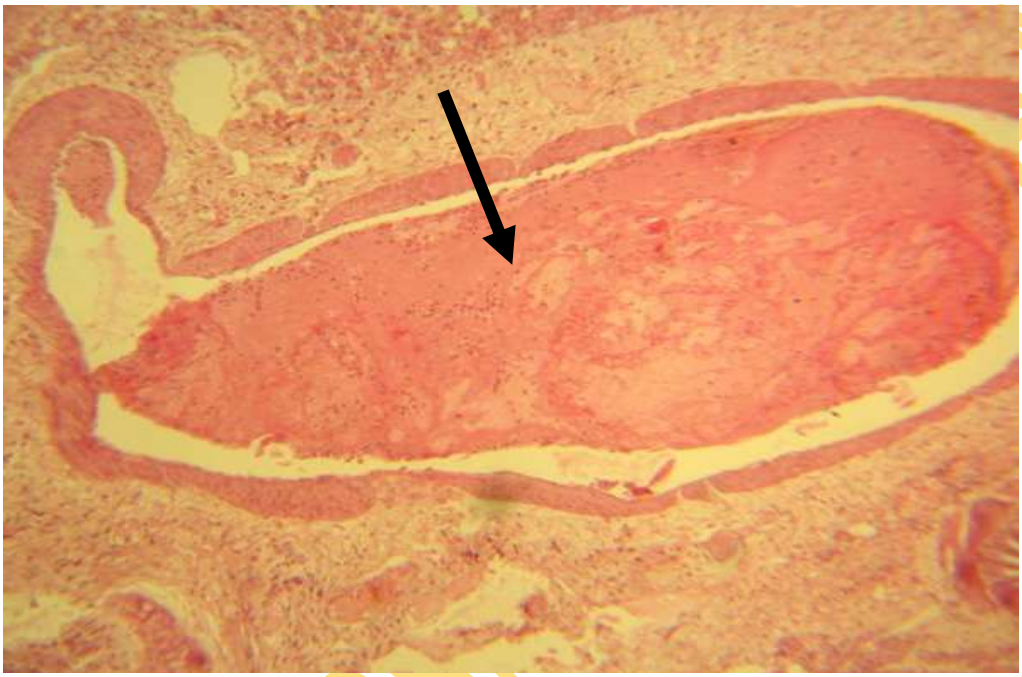

Fig. 6.30 Photomicrograph of the lung sections at day 3 p.i. showing pulmonary blood vessel with a thrombus attached to the vascular wall (arrows) H & E x 300.

Table 6.3.3 Pulmonary and the number of histopathological changes in guinea pigs infected intraperitoneally with *Leptospira interrogans* serovar *icterohaemorrhagiae.*

Lesions	**Contrl 10***	**½ day 4**	**day 1**	**day 3 4**	**day 5 3**	**day 7 3**	**Total (18)**	**(%)**	**Lower -Upper**
Pulmonary congestion and oedema	-	2	4	3	2	2	13	72.2	51.0 - 93.0
Pulmonary haemorrhage	-	-	1	3	3	2	09	50.0	27.0 - 73.0
Erythrophagocytosis	-	-	1	3	3	2	09	50.0	27.0 - 73.0
Haemosiderosis	-	-	-	2	3	2	07	38.9	16.0 - 62.0
Interstitial pneumonia	-	-	1	2	2	2	07	38.9	16.0 - 62.0
Activated alveolar macrophages	-	1	2	4	3	2	12	66.7	45.0 - 89.0
Thickened alveolar wall	-	1	4	4	3	3	15	83.3	66.0 - 100
Vascular thrombosis	-	-	-	1	2	3	06	33.3	11.0 - 55.0

Table 6.3.4 Semi-quantification of pulmonary histopathological changes in guinea pigs infected intraperitoneally with *Leptospira interrogans* serovar *icterohaemorrhagiae*.

Lesions	**Control**	½ day	**day 1**	day 3	day 5	**day 7**
Pulmonary congestion and oedema	-	++	++	++	+++	++
Pulmonary haemorrhage	-	-	+	++	+++	++
Erythrophagocytosis	-	+	+	+++	+++	++
Haemosiderosis	-	-	+	++	++	+++
Interstitial pneumonia	-	-	++	++	+++	+++
Activated alveolar macrophages	-	+	+	++	+++	+++
Thickened alveolar wall	-	+	+	++	+++	+++
Vascular thrombosis	-	-	-	+	++	+++

- = Absent, + = mild, ++ = moderate, +++ = marked or severe.

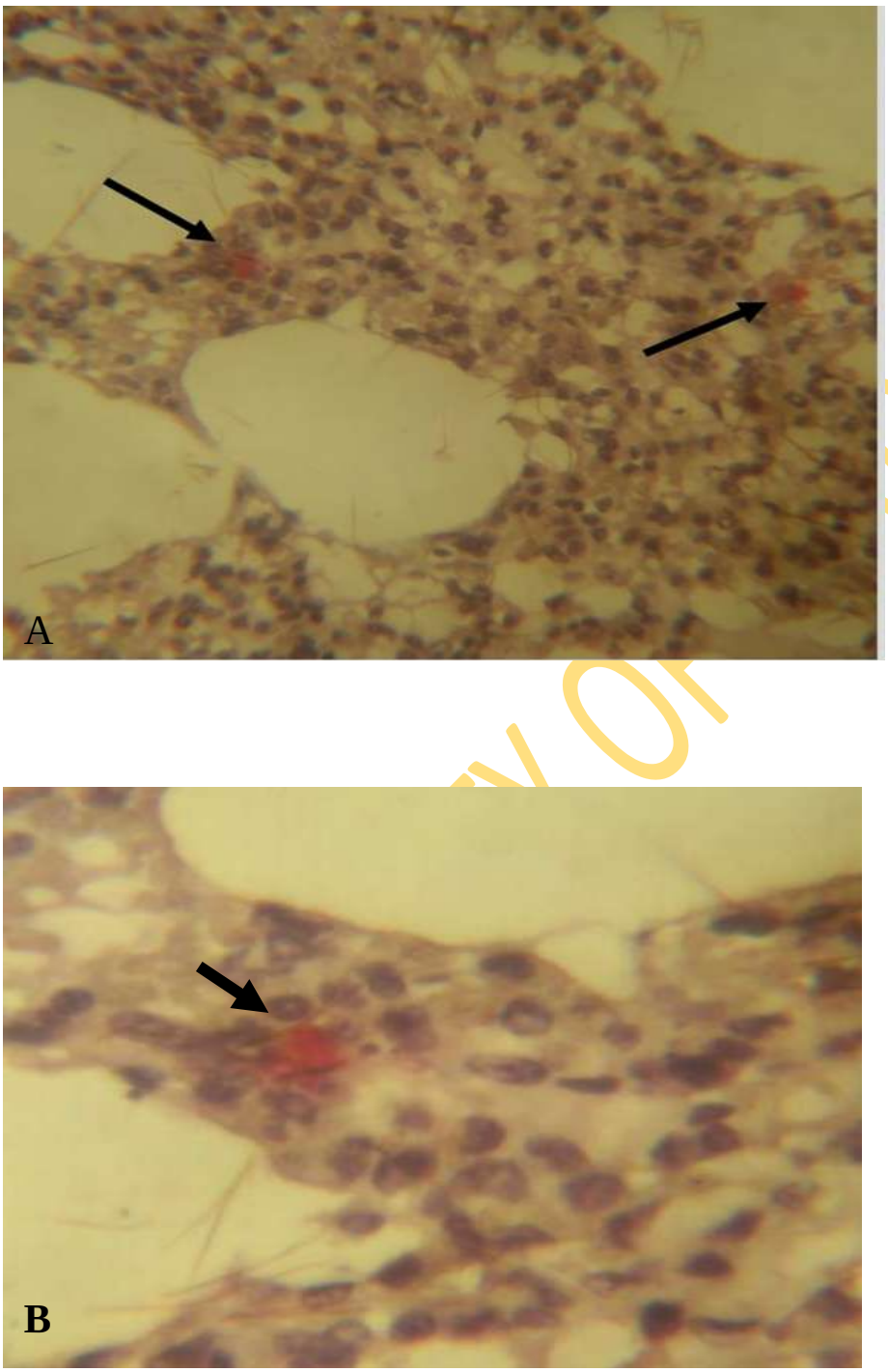

Fig. 6.31a & b Photomicrograph of alveolar wall showing two alveolar macrophages in the interstitium immunohistochemically demonstrating leptospiral antigen in their cytoplasm. Higher magnification of one of the alveolar macrophages in slide A. Alkaline phosphatase-fast red counterstained with haematoxylin, x 350.

6.6.3 . Renal histopathological changes in guinea pigs infected with *Leptospira icterohaemorrhagiae*

Table 6.3.5-6 and figures 6.32-34 depict renal histopathological changes in guinea pigs infected intraperitoneally with *Leptospira icterohaemorrhagiae*. The earlier lesions observed microscopically were moderate to severe congestion, interstitial and perivascular oedema with heamorrhages at 12hrs to day 3 p.i in 11 guinea pigs (fig.6.9.32). Mild to moderate tubular nephrosis and vacuolations were also present at day 1 to day 3 p.i. Moderate to severe tubular dilatation, hyaline cast, tubular nephrosis and interstitial mononuclear cells infiltration were more prominent at day 5 to day 7 p.i. The total renal histopathological changes such as congestion and oedema were observed in 12 (66.7%, 95%CI 45-89%) out of 18 animals in this group. Renal haemorrhages was present in 7 (38.9%, 95%CI 16 – 62%) animals, tubular nephrosis (being the highest highest histopathological alteration) in 13 (72.2%, 95%CI 51-93%) animals, tubular dilatation in 6 (33.3%, 95%CI 11-55%), and tubular hyaline cast and vacuolar degeneration were observed in 11 guinea pigs (61.1%, 95%CI 38-84%) each. Interstitial mononuclear cells infiltration was observed only in 2 animals (11.1%, 95%CI 0- 23%) at day 7 p.i. Immunohistochemical staining of the leptospiral antigen from interstitium via the tubular epithelial cells into the tubular lumen were also demonstrated (Fig.6.36a, b &c). Leptospiral organisms were demonstrated with Warthin Starry silver stain at day 5 p.i. within the tubular lumina (Fig 6.35). Granular forms of leptospire antigens were demonstrated immunohistochemically in the tubular lumina and cytoplasm of the tubular epithelial cells at day 5 p.i.

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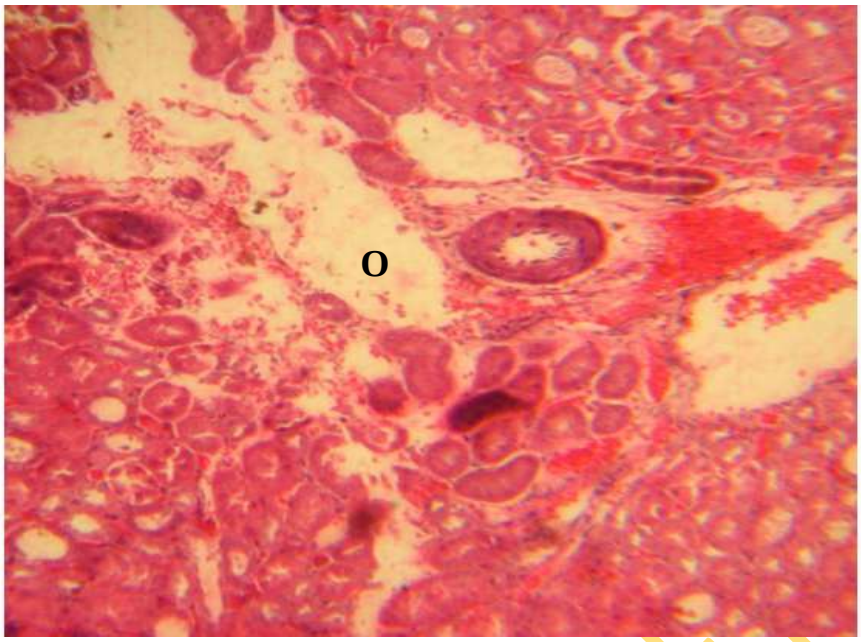


Fig. 6.32 Photomicrograph of kidney sections showing perivascular oedema (O) and interstitial haemorrhages at day 1p.i. H & E, x 250

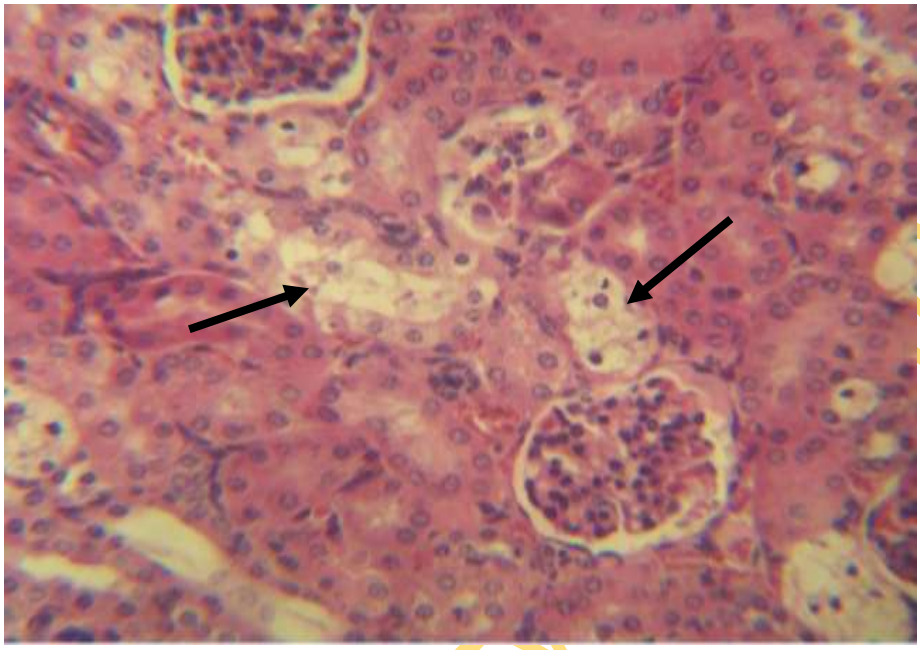


Fig. 6.33 Photomicrograph of kidney sections showing moderate multifocal tubular degeneration and necrosis (arrows) at day 3 p.i. H & E, x 350

Table 6.3.5 Renal histopathological alterations and the number of guinea pigs infected intraperitoneally with *Leptospira interrogans* serovar *Icterohaemorrhagiae*

Lesions	**Contrl (10*)**	**½ day (4)**	**day 1 (4)**	**day 3 (4)**	**day 5 (3)**	**day 7 (3)**	**Total (18)**	**%**	**Lower Upper**
Renal congestion and oedema	-	4	4	3	1	0	12	66.7	45.0 - 89.0
Renal haemorrhage	-	0	3	3	1	0	07	38.9	16.0 - 62.0
Tubular nephrosis	-	0	3	4	3	3	13	72.2	51.0 - 93.0
Tubular dilatation	-	0	0	2	1	3	06	33.3	11.0 - 55.0
Tubular hyaline cast	-	0	2	3	3	3	11	61.1	38.0 - 84.0
Vacuolar degeneration	-	0	2	3	3	3	11	61.1	38.0 - 84.0
Interstitial cellular infiltration	-	0	0	0	0	2	02	11.1	00.0 - 23.0

Table 6.3.6 Semi-quantification of renal histopathological alterations in guinea pigs infected intraperitoneally with *Leptospira interrogans* serovar *icterohaemorrhagiae.*

Lesions	**Control**	½ day	**day 1**	day 3	day 5	**day 7**
Renal congestion and oedema	+	++	+++	+++	+++	++
Renal haemorrhage	-	+	++	++	++	+
Tubular nephrosis	-	-	+	++	+++	+++
Tubular dilatation	+	-	-	++	++	++
Tubular hyaline cast	-	-	+	++	++	+++
Vacuolar degeneration	-	-	++	+++	+++	++
Interstitial cellular infiltration	-	-	-	-	-	+

- = Absent, + = mild, ++ = moderate, +++ = marked or severe.

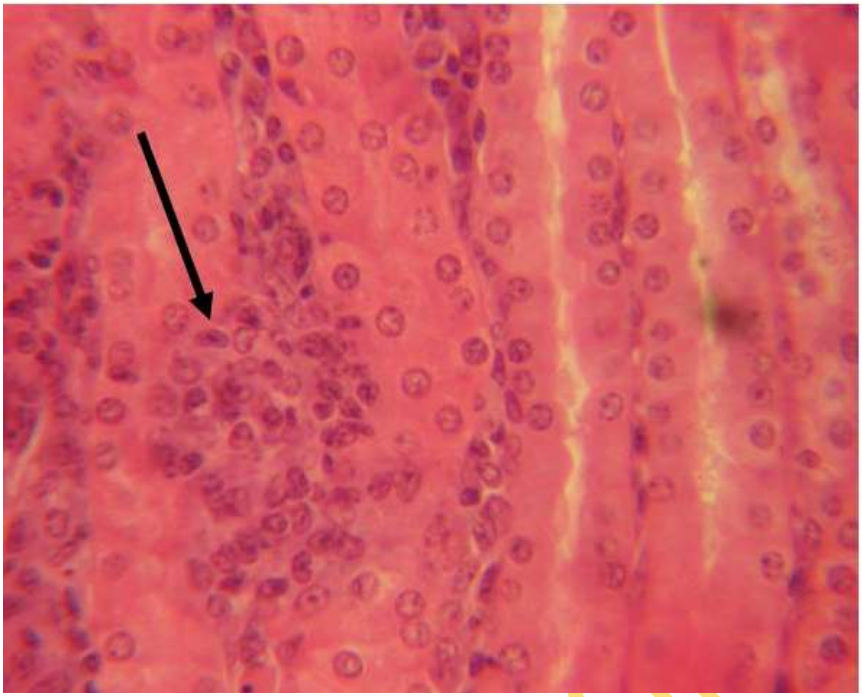


Fig. 6.34. Photomicrograph of kidney section showing interstitial lymphocytic cellular infiltration (arrow) at day 7 p.i. x 400. H & E

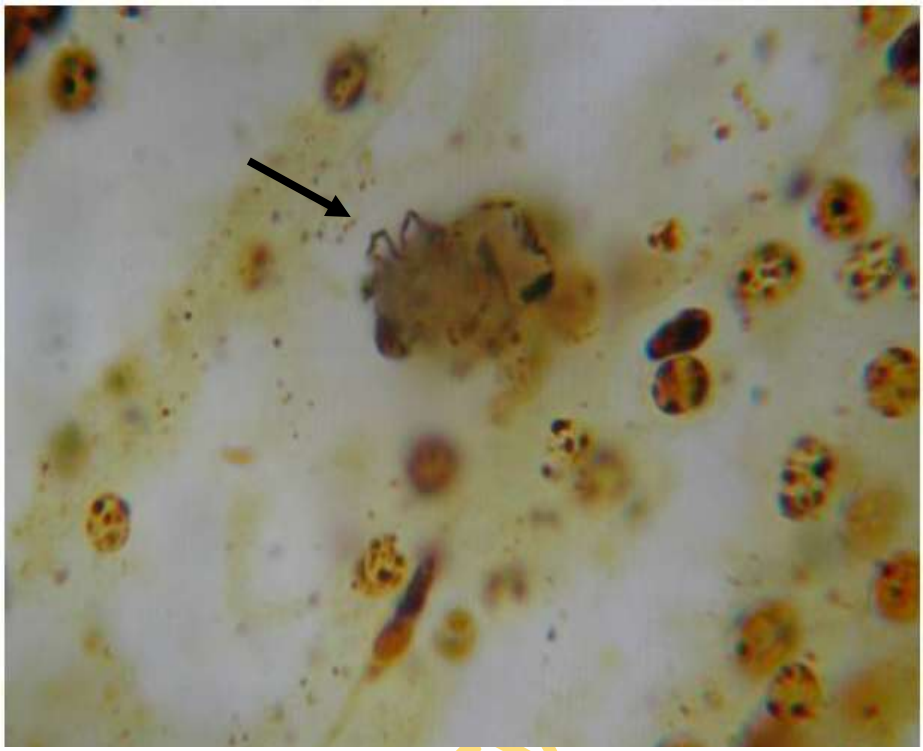


Fig. 6.35 Photomicrograph of the infected kidney section showing the presence of the leptospira organism at day 5 p.i. x 450 Warthin Starry silver stain.

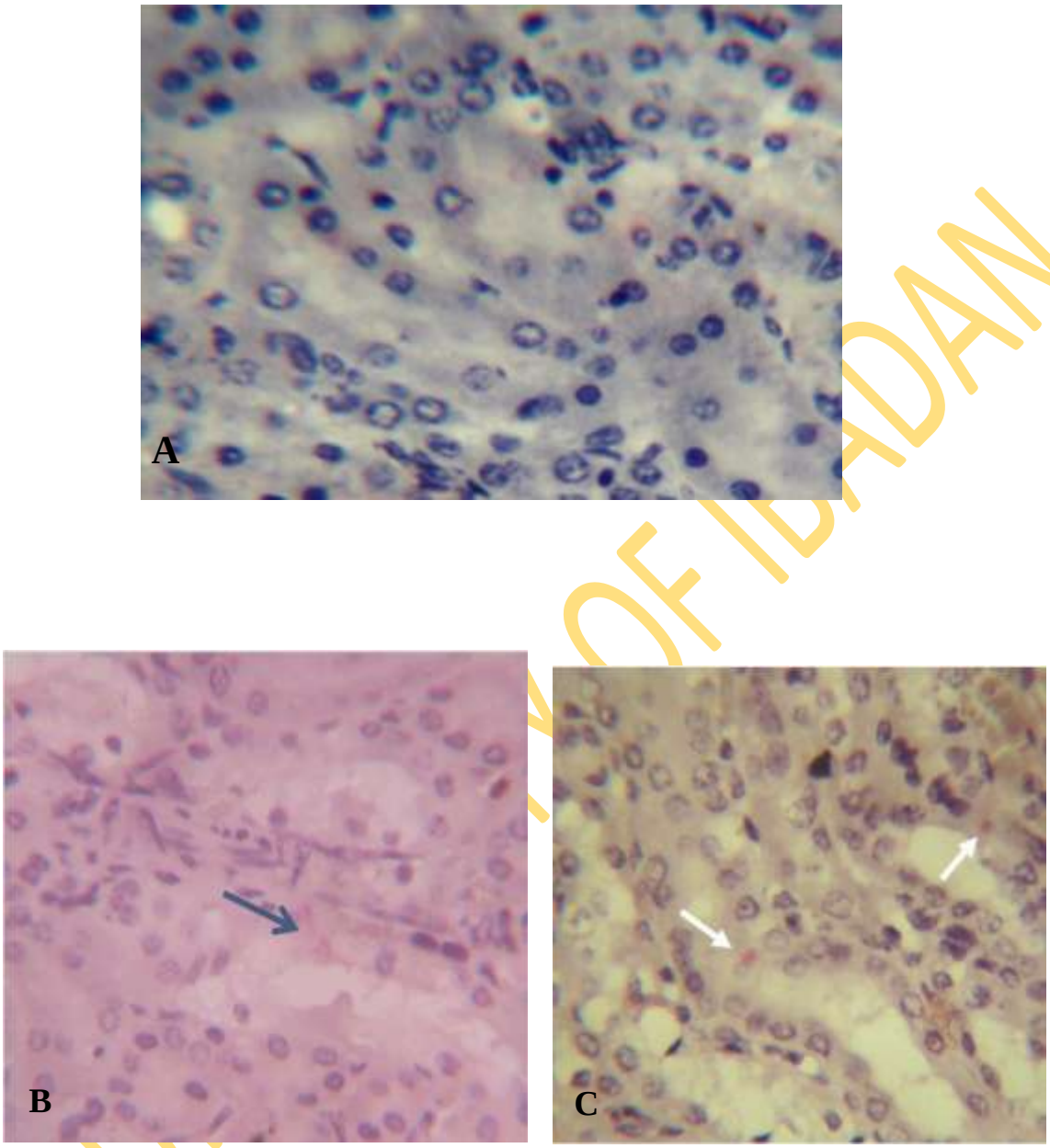
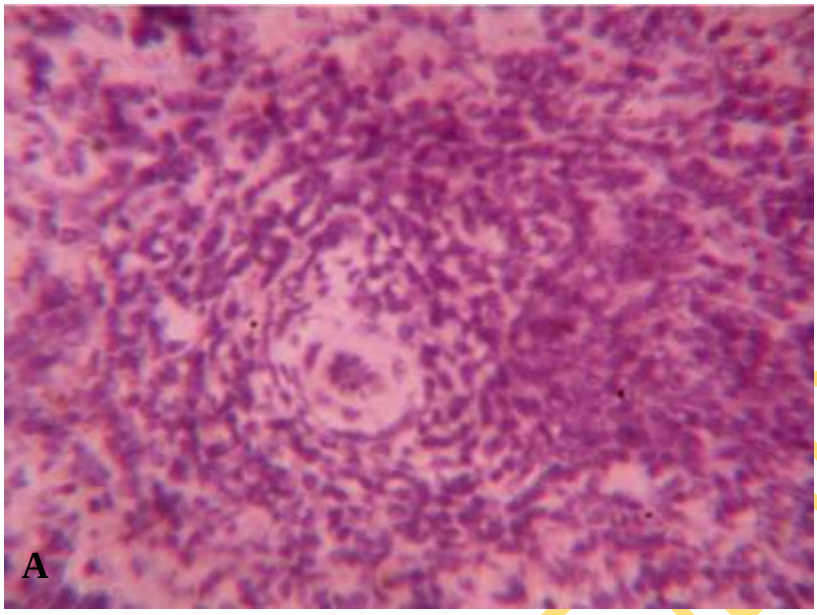
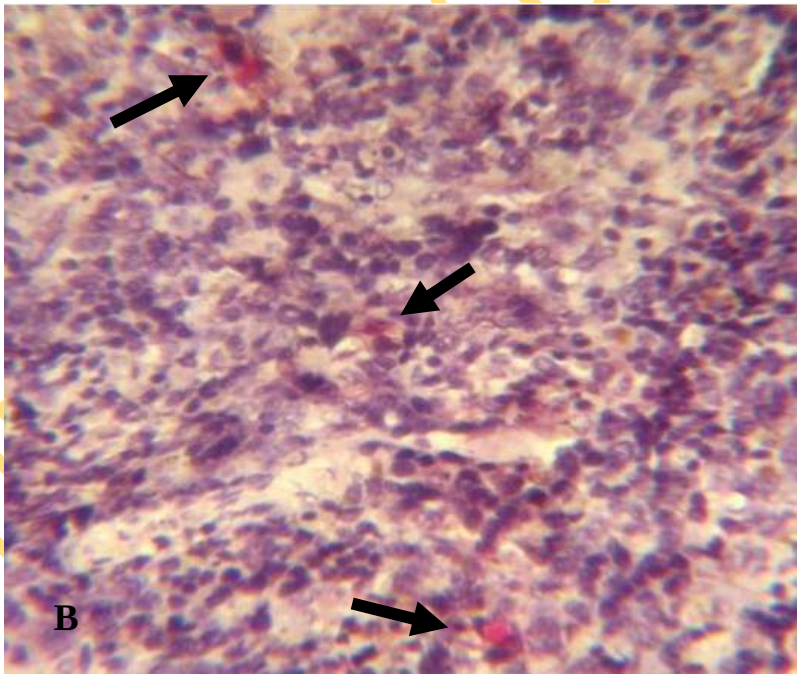


Fig. 6.36 a, b & c. Photomicrograph of kidney sections normal (A), presence of leptospiral antigen migrating from interstitium via the tubular epithelial cells at day 1 p.i (blue arrow) (B) and within tubular lumen and cytoplasm of the tubular epithelial cells at day 3 (C) x 300 Alkaline phosphatase-fast red counterstained with haematoxylin.

6.6.4 Splenic histopathological changes in guinea pigs infected with *Leptospira icterohaemorrhagiae*

Table 6.3.7-8 depicts splenic histopathological changes in guinea pigs infected intraperitoneally with *Leptospira icterohaemorrhagiae.* Various splenic histopathological changes were present in the infected guinea pigs compared with the control such as lymphoid hypoplasia in 13 (72.2% 95%CI 51-93%) animals, haemosiderosis in 16 (88.9% 95%CI 73-100%), erythrophagocytosis in 10 (55.6%, 95%CI 33-59%), erythroid hypoplasia in 11 (61.1% 95%CI 38-84%), sinusoidal hitocytosis in 13 (72.2% 95%CI 51-93%) and prominent trabeculae in 13 (72.2% 95%CI 51-93%) guinea pigs in this study. The severity of the histopathological changes progressed as the disease advances in the infected guinea pigs. Immunohistochemically, leptospire (granular form) were observed in the cytoplasm of reticuloendothelial cells (Figs.6.37a & b).





Figs. 6.37 a & b. Photomicrograph of the control spleen section negative control without immunoreactivity (A). x 300, and (B) showing the presence of leptospiral antigen in the cytoplasm of macrophages (arrows). Alkaline phosphatase-fast red counterstained with heamatoxylin. X 300

Table 6.3.7 Number and splenic histopathological alterations in guinea pigs infected intraperitoneally with *Leptospira interrogan* serovar *icterohaemorrhagiae.*

Lesions/ No of animals involved	Control (10)*	½ day (4)	day 1 (4)	day 3 (4)	day 5 (3)	day 7 (3)	Total (18)	(%)	95%CI Lower - Upper
Lymphoid hypoplasia	-	0	3	4	3	3	13	72.2	51.0 - 93.0
Erythroid hypoplasia	-	-	2	3	3	3	11	61.1	38.0 - 84.0
Histocytosis	-	-	3	4	3	3	13	72.2	51.0 - 93.0
Haemosiderosis	-	3	4	4	3	2	16	88.9	73.0 - 100
Erythrophagocytosis	-	0	2	4	3	1	10	55.6	33.0 - 59.0
Prominent trabeculae	-	0	3	4	3	3	13	72.2	51.0 - 93.0

Table 6.3.8 Semi-quantification of the splenic histopathological alterations in guinea pigs infected intraperitoneally with *Leptospira interrogans* serovar *icterohaemorrhagiae*.

Lesions	**Control**	½ day	**day 1**	day 3	day 5	**day 7**
Lymphoid hypoplasia	-	-	++	++	+++	+++
Erythroid hypoplasia	-	-	+++	+++	+++	+++
Histocytosis	-	-	+++	++	++	+++
Haemosiderosis	+	+	+	+	++	++
Erythrophagocytosis	-	-	+	++	++	++
Prominent trabeculae	-	-	++	++	++	+++

- = Absent, + = mild, ++ = moderate, +++ = marked or severe.

6.6.5 Myocardial histopathological changes in guinea pigs infected with *Leptospira icterohaemorrhagiae*

The histopathological changes in the hearts of the infected guinea pigs are depicted in Table 6.3.8-9 and Figures 6.38-39. Mild to severe myocardial congestion and interstitial oedema were consistent findings at 12hrs to day 7 p.i.in 13 (72.2% 95%CI 51-93%) and 16 (88.9% 95%CI 73-100%) respectively, while mild to moderate myocardial necrosis and mononuclear cells infiltration (mostly lymphocyte and plasma cells) were more prominent at day 7 p.i in 11 (61.1% 95%CI 38-84%) and 7 (38.9% 95%CI 16 - 62%) guinea pigs respectively. Immunohistochemical staining of leptospira antigens was not observed in all the heart section examined.

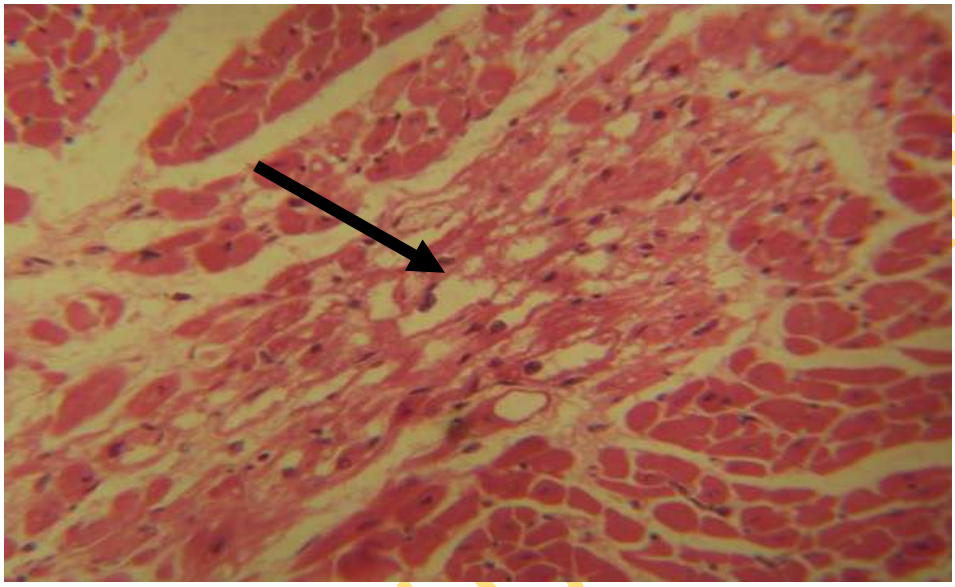


Fig. 6.38 Photomicrograph of the heart section showing moderate diffuse interstitial oedema and a focal area of myocardial vacuolations and necrosis (arrow) at 72hrs p.i. H & E, x 350.

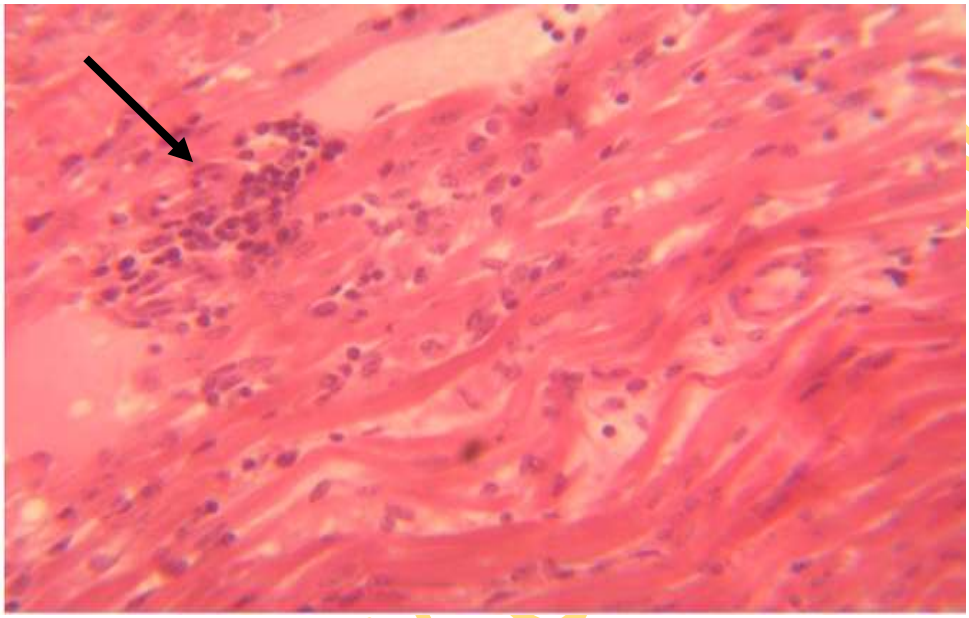


Fig. 6.39. Photomicrograph of the heart section showing moderate diffuse interstitial inflammatory oedema, myocardial necrosis and locally extensive area of mononuclear cellular infiltration (arrow), mostly lymphocyte and macrophages at day 7 p.i ., x 400, H & E.

Table 6.3.9 Number and myocardial histopathological changes in guinea pigs infected intraperitoneally with *Leptospira interrogans* serovar *icterohaemorrhagiae.*

Lesions/ Time and number of animals involved	**Control (10)**	**½ day (4)**	**day 1 (4)**	**day 3 (4)**	**day 5 (3)**	**day 7 (3)**	**Total (18)**	**(%)**	**Lower - Upper**
Congestion	-	4	3	3	2	1	13	72.2	51.0 - 93.0
Interstitial oedema	-	4	4	3	3	2	16	88.9	73.0 - 100
Myocardial necrosis	-	-	2	3	3	3	11	61.1	38.0 - 84.0
Lymphoplasmacytic infiltration	-	-	1	2	2	2	07	38.9	16.0 - 62.0

Table 6.3.10 Semi-quantification of myocardial histopathological changes in guinea pigs infected intraperitoneally with *Leptospira interrogans* serovar *icterohaemorrhagiae*.

Lesions	**Control**	½ day	**day 1**	day 3	day 5	**day 7**
Congestion	-	++	++	++	+	+
Interstitial oedema	-	++	++	++	++	+++
Myocardial necrosis	-	-	+	++	++	++
Lymphoplasmacytic infiltration	-	-	-	++	++	++

- = Absent, + = mild, ++ = moderate, +++ = marked or severe

6.6.6 Adrenal histopathological changes in guinea pigs infected with *Leptospira icterohaemorrhagiae*

Table 6.3.11-12 and Figures 6.40a & b shows the adrenal histopathological changes in infected guinea pigs. The adrenal blood vessels were slightly congested at the commencement of the study, but were moderately congested at day 1 to day 3 post infection in 14 (77.8%, 95%CI 59 – 97%) guinea pigs. Moderate to severe difusse vacuolar degeneration were observed in the three layers of adrenal gland, with zona glomerulosa being most severely affected in 16 (88.9%, 95%CI 73 - 100%) animals. The zona glomerulosa showed moderate disorganization of its cellular arrangement.

Table 6.3.11 Adrenal histopathological alterations in guinea pigs infected intraperitoneally with *Leptospira interrogans* serovar *icterohaemorrhagiae.*

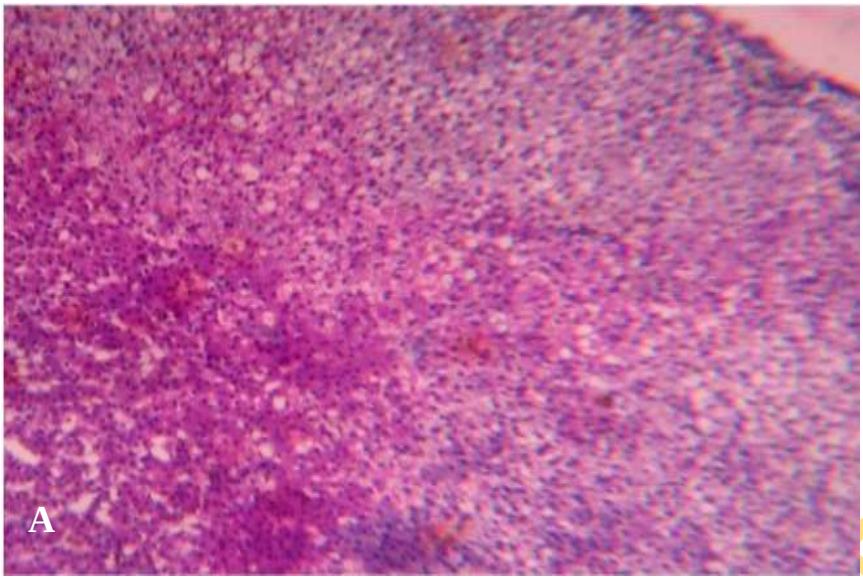
Lesions/ No of animals involved	**Control (10)***	**½ day (4)**	**day 1 (4)**	**day 3 (4)**	**day 5 (3)**	**day 7 (3)**	Total (**18**)	**(%)**	**Lower - Upper**
Congestion	-	3	3	4	2	2	14	77.8	59.0 - 97.0
Vacuolar degeneration	7	2	4	4	3	3	16	88.9	73.0 - 100
Zona glomerulosa disorganization	-	1	3	3	3	3	13	72.2	51.0 - 93.0

- = Absent.

Table 6.3.12 Semi-quantification of adrenal histopathological alterations in guinea pigs infected intraperitoneally with *Leptospira interrogans* serovar *icterohaemorrhagiae*.

Lesions	**Control**	½ day	**day 1**	day 3	day 5	**day 7**
Congestion	-	+	++	++	+	+
Vacuolar degeneration	+	++	+++	+++	++	+++
Zona glomerulosa disorganisation	-	-	++	++	++	++

- = Absent, + = mild, ++ = moderate, +++ = marked or severe



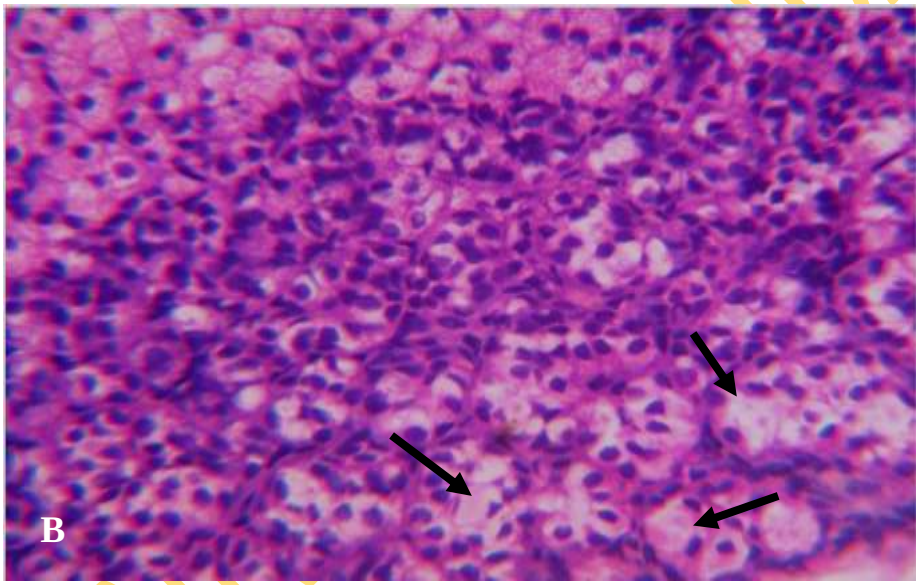


Fig. 6.40 a & b. Photomicrograph of normal adrenal gland (A) and B showing moderate diffuse vacuolar degeneration of the zonal glomerulosa (arrows) in leptospiral infected guinea pig at 24 hrs p.i. x 350. H & E.

6.6.7 Pancreatic histopathological changes in guinea pigs infected with *Leptospira icterohaemorrhagiae*

Table 6.3.13-14 and figures 6.41a & b. depicted the histopathological changes in the pancreas of infected guinea pigs. Mild to moderate vascular congestions were observed throughout the period of this study in 17 (94.4% 95%CI 83 – 100%) guineas pigs, but were more prominent at 12hrs to day 3 post infection. But, there were moderate to severe interstitial oedema and haemorrhages at day 3 – 7 post infection in 13 (72.2% 95%CI 51-93%) and 3 (16.7%, 95%CI 0 - 34%) animals respectively. Mild pancreatic degeneration and necrosis, with sparse mononuclear cell infiltration in the interstitium were observed only at day 3-7 post infection in 6 (33.4% 95%CI 11-55%) and 3 (16.7% 95%CI 0 - 34%) guinea pigs respectively.

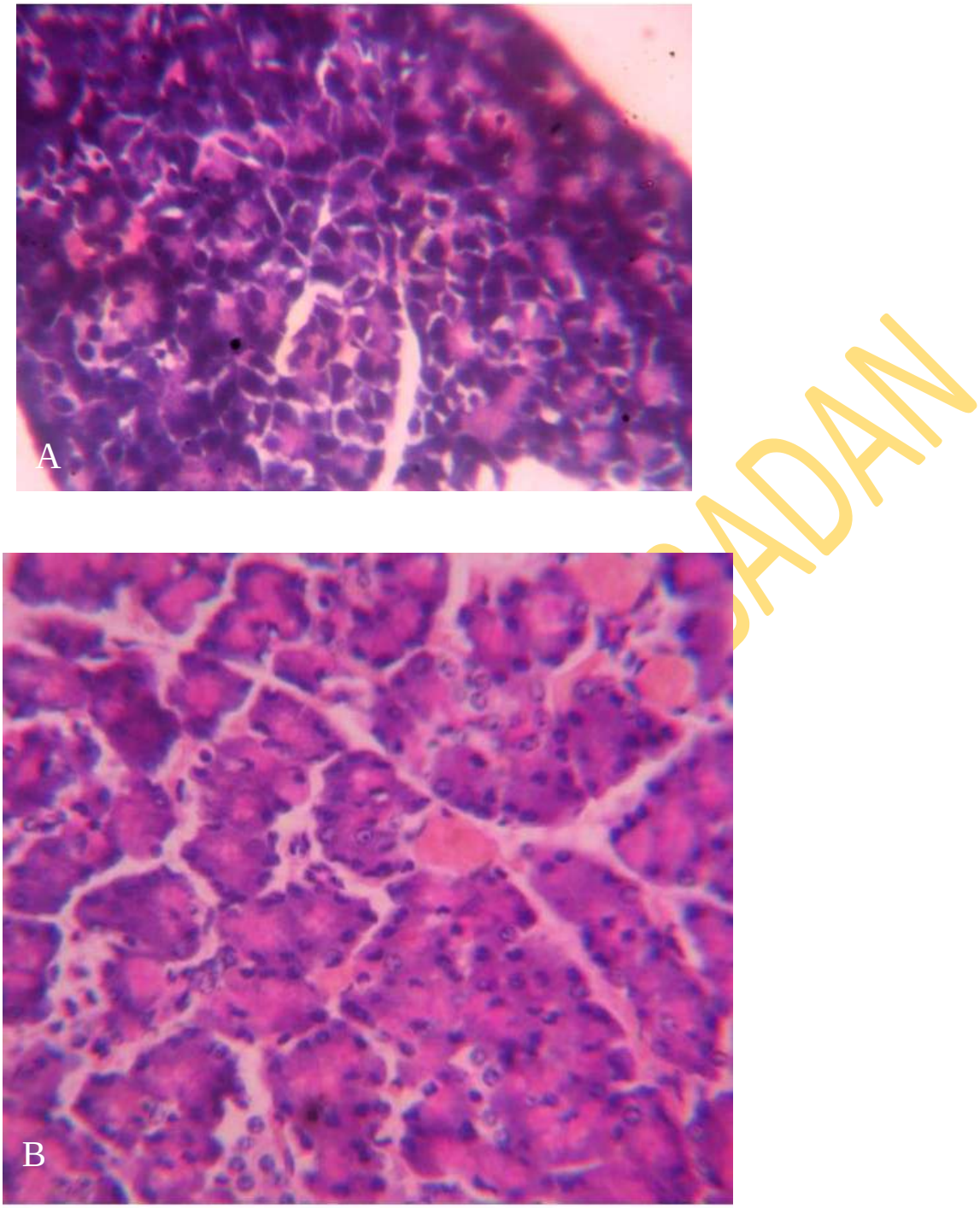


Fig.6.41a & b Photomicrograph of normal pancreas (A), and (B) showing prominent interstitial oedema with sparse interstitial mononuclear cells infiltration . H & E. x350.

Table 6.3.13 Pancreatic histopathological alterations in guinea pigs infected intraperitoneally with *Leptospira interrogans* serovar *icterohaemorrhagiae*.

Pancreatic lesions	Control (10)*	½ day (4)	day 1 (4)	day 3 (4)	day 5 (3)	day 7 (3)	Total (18)	(%)	Lower - Upper
Vascular congestion	-	4	4	4	1	0	17	94.4	83.0 – 100.0
Interstitial oedema	-	4	4	2	2	1	13	72.2	51.0 - 93.0
Interstitial haemorrhage	-	0	1	1	1	0	3	16.7	00.0 – 34.0
Pancreatic degeneration	-	0	0	2	2	2	6	33.4	11.0 – 55.0
Interstitial lymphocytic infiltration	-	0	0	0	1	2	3	16.7	00.0 - 34.0

Table 6.3.14 Semi-quantification of pancreatic histopathological alterations in guinea pigs infected intraperitoneally with *Leptospira interrogans* serovar *icterohaemorrhagiae*.

Lesions	**Control**	½ day	**day 1**	day 3	day 5	**day 7**
Vascular congestion	-	++	++	++	+	+
Interstitial oedema	-	+	++	++	++	+++
Interstitial haemorrhage	-	-	++	++	++	++
Pancreatic degeneration	-	-	-	+	+	+
Interstitial lymphocytic infiltration	-	-	-	-	+	+

- = Absent, + = mild, ++ = moderate, +++ = marked or severe

6.6.8 Cerebral histopathological changes in guinea pigs infected with *Leptospira icterohaemorrhagiae*

The cerebral histopathological changes in the leptospiral infected guinea pigs are depicted in Table 6.3.15-16 and Figures 6.42-45. There was moderate to severe menigeal congestion in 12 (66.7% 95%CI 45 - 89%) with mild to moderate perivascular oedema and nonsuppurative perivascular cuffing from day 1 to day 5 post infection in 16 (88.9% 95%CI 74-100%) guinea pigs (Figs.6.9.42-43). Mild to moderate endothelial cell swelling and blebs in 11 (61.1% 95%CI 38 - 84%) animals were observed. Moreso, moderate to marked neuronal degeneration and gliosis were the prominent features at 24 hrs - day 7 p. i in 9 (50% 95%CI 27 - 73%). Immunoreativity to leptospire antigens were observed in the endothelial cells, cerebral parenchyma and within the cytoplasm of the glial cells at 12hrs – day 3 post infection (Figs.6.44a & b-45).

Table 6.3. 15 Cerebral histopathological alterations in guinea pigs infected intraperitoneally with *Leptospira interrogans* serovar *icterohaemorrhagiae.*

Lesions	**Control (10*)**	**½ day (4)**	**day 1 (4)**	**day 3 (4)**	**day 5 (3)**	**day 7 (3)**	Total **(18)**	**(%)**	**Lower - Upper**
Meningeal congestion	-	4	4	1	2	1	12	66.7	45.0 – 89.0
Perivascular oedema and cuffing	-	4	4	4	2	2	16	88.9	74.0 – 100.0
Endothelial swelling	-	4	4	2	1	0	11	61.1	38.0 – 84.0
Neuronal degeneration and gliosis	-	0	1	2	3	3	09	50.0	27.0 – 73.0

Table 6.3.16 Semi-quantification of cerebral histopathological alterations in guinea pigs infected intraperitoneally with *Leptospira interrogans* serovar *icterohaemorrhagiae.*

Brain lesions	**Control**	½ day	**day 1**	day 3	day 5	**day 7**
Meningeal congestion	-	++	+++	+++	+ +	+
Perivascular oedema and cuffing	-	++	++	++	++	+
Endothelial swelling	-	+	++	+	-	-
Neuronal degeneration and gliosis	-	-	++	++	+++	+++

- = Absent, + = mild, ++ = moderate, +++ = marked or severe

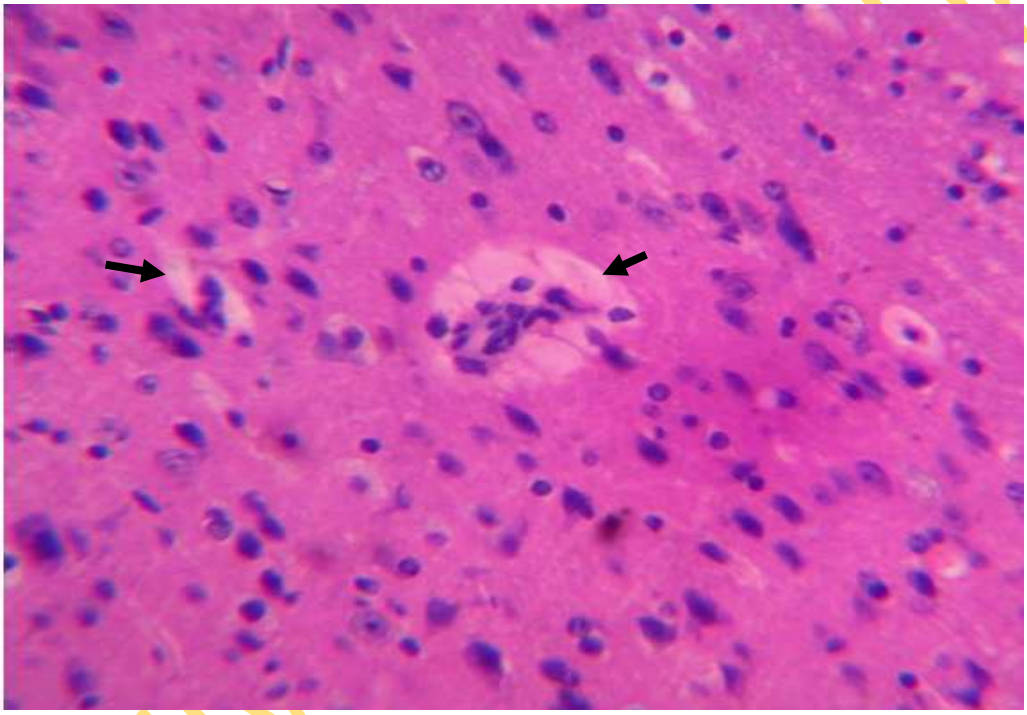


Fig. 6.42 Photomicrograph of the brain of leptospira infected guinea pig showing perivascular oedema (arrows) at day 1 p.i. x 300, H & E.

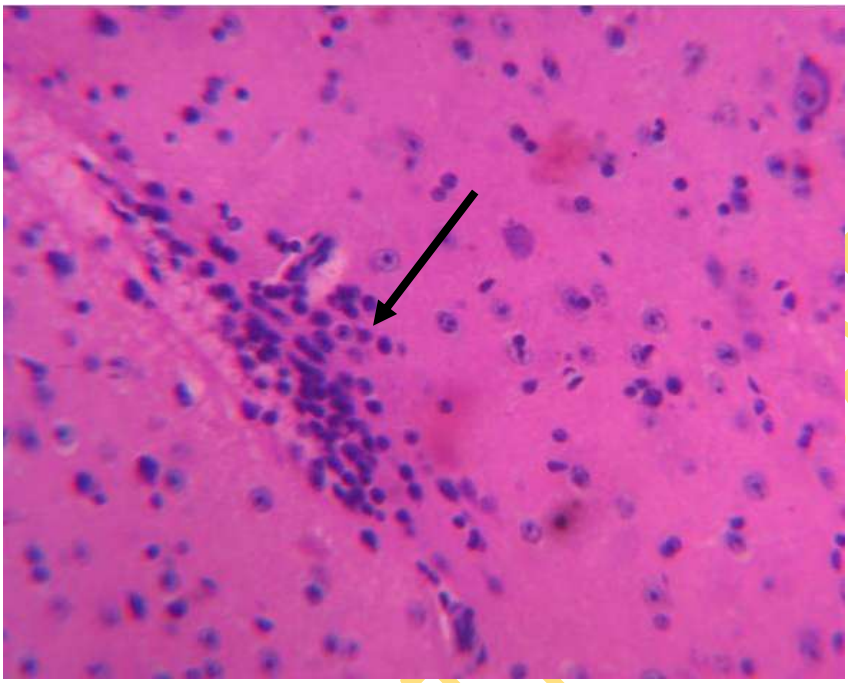


Fig. 6.43 Photomicrograph of the brain of leptospiral infected guinea pig showing focal area of perivascular cuffing (arrow) with mild gliosis. x 300, H & E.

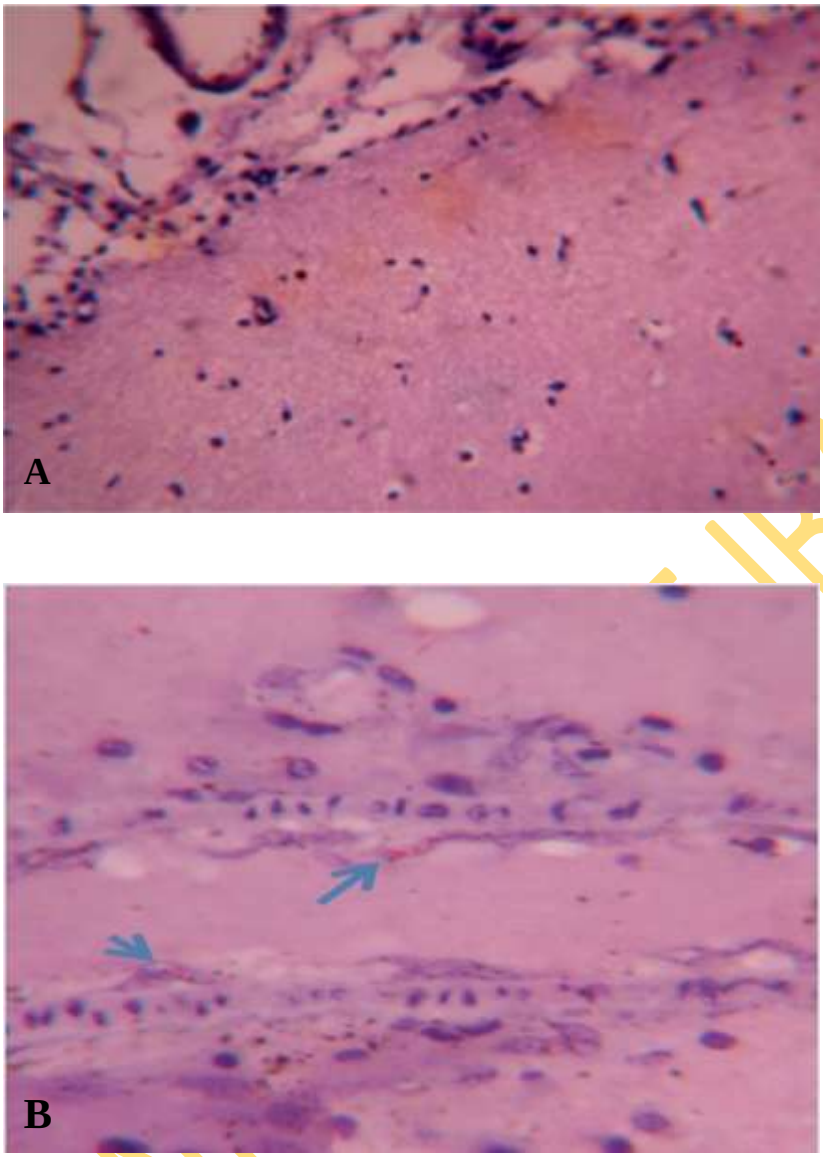


Fig. 6.44a & b Photomicrograph of the brain of guinea pigs showing normal control (A) and immunoreactivity to the leptospiral antigens in the endothelial cells (blue arrows) at 12hrs p.i (B). Alkaline phosphatase-fast red counterstained with heamatoxylin. x 300

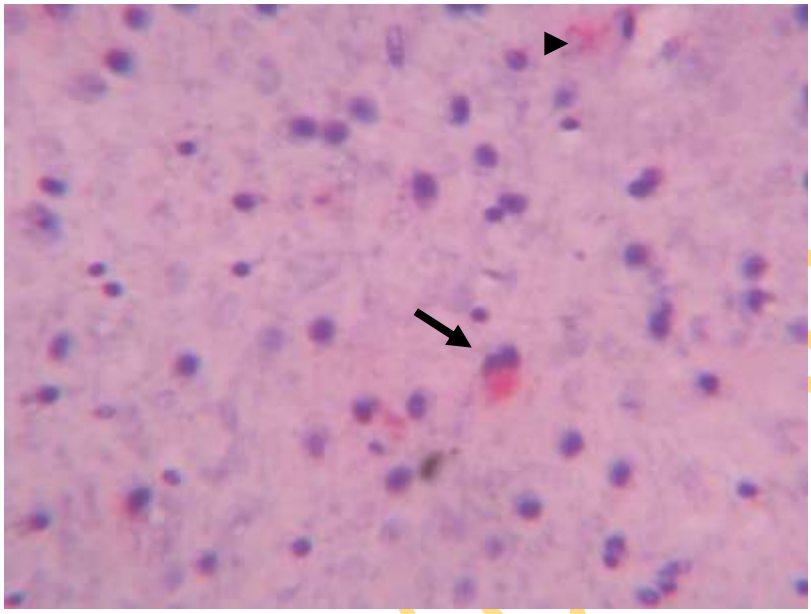


Fig. 6.45 Photomicrograph of the brain of guinea pigs showing immunoreactivity to the leptospiral antigens within glial cells (black arrow) and the cerebral tissue (head arrow) of the brain at day 1 p.i. x 350. Alkaline phosphatase-fast red counterstained with heamatoxylin.

Table 6.4 Comparative results of the four diagnostic techniques for the detection of leptospiral organism and the presence of microscopic lesions in different organs of infected guinea pigs.

	EMJH Isolation	IHC	WSS	PCR	Lesions
Organs					
Kidney	++	+	++	+	+
Lung	++	+	-	+	+
Liver	++	+	-	ND	+
Brain	++	+	-	++	+
Adrenal gland	++	-	-	++	+
Spleen	++	+	-	+	+
Pancreas	+ +	-	-	+	+
Heart	++	-	-	+	+

Table 6.4.1 Comparative diagnostic summary of leptospiral organism with time, in guinea pigs experimentally infected with *L. interrogans* serovar *icterohaemorrhagiae*, using EMJH medium, polymerase chain reaction (PCR) and immunohistochemistry

	Heart			**Liver**			**Kidney**			**Spleen**			**Lung**			**Pancreas**			**Adrenal gld**			**Brain**		
Duration	cul	im	pcr	cul	im	pcr	cul	im	pcr	cul	im	pcr	cul	im	pcr	cul	im	pcr	cul	im	pcr	cul	im	pcr
½ Day	-	-	+	+	+	ND	+	+	+	+	+	-	+	+	+	+	-	+	+	-	+	+	+	+
Day 1	+	-	-	+	+	ND	+	+	+	+	+	-	+	+	+	+	-	ND	+	-	ND	+	+	-
Day 3	-	-	-	+	+	ND	+	+	+	-	-	+	+	+	-	-	-	+	+	-	+	-	-	-
Day 5	-	-	-	+	+	ND	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Day 7	-	-	-	-	-	ND	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

ND = Not determined, - = absent, + = present, cul = Culture in EJHM, im= immunohistochemistry, pcr = polymerase chain reaction

Table 6.4.2 Comparative summary of histopathological changes and isolation of leptospiral organism in naturally-infected dogs and experimentally-infected guinea pigs model.

	Dog	**Guinea pigs**
Organs/Morphological diagnosis	**Lesions (leptospiral isolation)**	**Lesions (leptospiral detection)**
Lungs		
Pulmonary congestion, oedema and haemorrhages	**Present (-)**	Present (+) 12hrs – day 5 p.i.
Kidney		
Tubular nephrosis and interstitial nephritis	**Present (+)**	**Present (+) 12hr- day 7 p.i.**
Pancreas		
Acute haemorrhagic pancreatitis	**Present (-)**	Present (+) 12hrs – day 3 p.i.
Adrenal gland		
Vacuolar degeneration	**ND**	Present (+) 12hrs – day 3 p.i.
Brain		
Non-suppurative encephalitis	**Present (-)**	Present (+) 12hrs – day 1 p.i.
Spleen		
Splenic hypoplasia	**Present (-)**	Present (+) 12hrs – day 3 p.i.
Heart		
Non-suppurative myocarditis	**Present (-)**	Present (+) 12hrs – day 1 p.i.
Liver		
Foci of hepatic necrosis and loss of cohesion	**Present (±)**	Present (+) 12hrs – day 5 p.i.

+= from 12hrs to day 1 p.i. (Brain, heart).
+= from 12hrs to 3rd day p.i (Pancreas, spleen, adrenal gland).
+= from 12hrs to day 5 p.i. (Liver, lung).
+= from 12hrs to day 7p.i. (Kidney).

Table 6.4.2 shows that the dissemination and clearance of leptospiral organism from different organs of infected guinea pigs were time-dependent.

6. 7. DISCUSSION

Experimental leptospirosis in guinea pigs has been characterized with clinical signs of dullness, weakness, anorexia, hemorrhages, conjunctivitis, anemia and severe jaundice (Abdulkader, 1997). These clinical manifestations are similar to those observed in this study. In this study, analysis of the haematological indices demonstrated that there seem not to be severe anemia following experimental infection of guinea pigs with *leptospira interrogans* serovar *icterohaemorrhagiae.* The seemingly normal hematological indices in this study demonstrated initial normocytic normochromic anemia that progressesed to microcytic normochromic anemia and later microcytic hypochromic anemia. These findings were similar to the work of Nally *et al.*, (2004), but the microcytic hypochromic anemia at day 5 p.i. observed in this study was not documented in their report. These findings might be due to persistent haemorrhages at the beginning which resulted to iron deficiency or lack of erythropoietin caused by the damage inflicted on the juxtaglomerular apparatus in the kidney by leptospiral infection in this study.

The cause of high levels of MCHC at day1-3 p.i might have been due to haemolysis or haemoconcentration due to dehydration. Despite the significant high levels of in vivo MDA at day 2 and day 3 p.i, there was no significant correlation with the PCV and the in vivo MDA, which further corroborate the normocytic- microcytic type of anaemia observed in this study.

The anaemia observed in *leptospira icterohaemorrhagiae* has been attributed to tissue hemorrhages and a decreased production of erythrocytes rather than an increased destruction of red blood cells (Low, 1964). Various potential virulent factors have been identified in pathogenic leptospire organism to attack various cells and tissues.

These include haemolysins, sphingomyelinase C, sphingomyelinase H, and haemolysis-associated protein-1. The relevance of these potential virulent factors in the pathogenesis of anaemia has not been elucidated. Also, the role of oxidative bursts products from neutrophils and activated monocytes produced during leptospiral infection which have the capability of initiating oxidative damage to the polyunsaturated fatty acid components of erythrocyte plasma membrane leading to cell destruction (Taiwo *et al.,* 2003) in the pathogenesis of anaemia has not also been elucidated.

In this study, the in vitro lipid peroxidation of erythrocytes challenged by hydrogen peroxide was not significant. Lipid peroxidation has been shown to cause an increase in the stiffness and deformity of the red blood cell membrane, which partially increases the susceptibility to hemolysis (Harvey, 1997). The slight increased production of in vitro lipid peroxidation in infected guinea pigs demonstrated the ability of the animal to prevent lipid peroxidation in the erythrocyte membrane. This was demonstrated by the negatively not significant correlation observed between the PCV and the in vitro lipid peroxidation in this study. Furthermore, the result of this study shows that lipid peroxidation might not have contributed significantly to the anaemia observed in leptospirosis. This was demonstrated in the non-significant positive correlation between the in vivo and in vitro lipid peroxidation.

The insignificant negative correlation between the in vitro lipid peroxidation and the PCV values with non-responsive type of anaemia suggests that lipid peroxidation might not have played a significant role in the pathogenesis of anaemia in leptospiral infected guinea pigs.

The normal levels of total protein, albumin, globulin and ALP from 12hrs to day 7 p.i. in this study were in agreement with the findings of Nally *et al.*, (2004) who infected guinea pigs with *leptospire interrogans* serovar *Copenhageni*, but there was significant increase in ALP levels in their study. Although, there was no significant change in the mean values of

bilirubin in this study, but there was slight increase in the levels of bilirubin. This is in agreement with the works of Lourdault *et al*., (2009) who observed no significant change in the mean value of bilirubin in the first 5 days p.i.compared with the non infected guinea pigs.

The increases in the BUN at 12hrs to day 1 p.i. without corresponding increase in the mean values of creatinine suggest early pre-renal azotemia. The cause of this pre-renal azotemia appeared to be obscured, but it is possible to suggest that early dissemination of leptospires to different organs which induces various injuries might have been responsible for this change. This is also corroborated by the hyperkalemia observed at this period (12hrs to day 1p.i.) which might be due to transcellular shift of K+ ions from intracellular fluid to extracellular fluid (George, 2003).

The slight to moderate elevated levels of urea and creatinine on the 5th and 7th days' p.i. might probably suggest renal azotemia due to tubular nephrosis, presence of leptospire and interstitial nephritis observed at this period. This is in agreement with other studies elsewhere (Lourdault *et al*., 2009). Although, these changes might not have been sufficient to induce renal failure in these animals, but prolong anorexia suffered by these animals might have contributed to the azotemia.

Different authors have used various methodologies such as dark-field microscopy, transmission electron microscopy, immunoflorence, immunohistochemistry, quantitative PCR, Warthin Starry silver stain and Levaditti's stain to study the pathogenesis of leptospirosis (Faine, 1957a,b; Marshall, 1976; Sitprija *et al*., 1980; Merien *et al*., 1998, Athanazio *et al*., 2008; Lourdault *et al*., 2009) in different animal model such as mice, hamster Wistar rats and guinea pigs. In this study, culture isolation, polymerase chain reaction, immunohistochemistry and Warthin Starry silver stain were used to study the dissemination kinetics of leptospirosis in various organs of guinea pigs model. The use of four different methodologies such as culture isolation, PCR, WSSS, and

immunohistochemistry appears to be rare in the study of leptospire dissemination kinetics in guinea pigs model in the literature. Although, Nally *et al.,* (2005a) used immunohistochemistry, immunoflorence and electron microscopy, but organs such as heart, pancreas, brain and adrenal gland were not examined in their report.

In humans, leptospires have been detected in the cerebellum and cerebrospinal fluid in patients with leptospirosis (Romero *et al*., 1998; Brown *et al.,* 2003). In guinea pig model, Lourdault *et al*., (2009) failed to detect significant number of leptospires in the brain of animals infected with l*eptospire interrogans Fiocruz L1-130* strain, collected at day 6 p.i. They suggested that a prolong period of infection might be necessary for leptospire to invade the cerebral parenchyma. This is not in agreement with this study, in which leptospires were detected by culture, PCR and immunohistochemistry as early as 12hrs to day 1 p.i. This appears to be a rare finding in the literature. Possibly, in the study of these workers (Lourdault *et al*., 2009), it is possible that leptospire invasion of the brain tissue might have taken a brief and unnoticed period (between 12 hrs to day 1 p.i) which made the organism to be undetectable throughout the period of their study.

There is a consensus in the literature on the haematogenous pathway of leptospiral organism to different organs and tissues, especially kidney. The organism enters the interstitium through the endothelial cells of the vascular wall (Marshall, 1976; Stripriya *et al.,* 1980) and this was referred to as the acute phase of the disease. Various authors have also demonstrated the presence of the organism in the vascular wall and within the interstitium by different methodologies. Marshall, (1976) detected leptospire organism within the interstitium at day 2 p.i. using transmission electron microscope, Striprija *et al.,* (1980), Merien *et al.,* (1998) and Athanazio *et al.*, (2008) used immunoflorence to detect leptospires at 3-4hrs, day 1 and 3 p.i. respectively in the interstitium. This study was in agreement with the works of Merien *et al.,* (1998) who detected leptospire in the kidney at day 3 p.i., although with different

methodology. But the demostration of leptospire organism from the interstitium to the tubular lumina in an experimental model, using immunohistochemistry, appears to be a rare finding in the literature. In this study, leptospire was observed at day 1 p.i within the tubular wall migrating into the tubular lumina.

Although, few cases of acute pancreatitis has been documented in humans (Kaya *et al.*, 2005), but none of these workers have been able to isolate the organism from the pancreas. Reports on the involvement or dissemination of leptospiral organism into the pancreas during acute phase of the infection in laboratory animal model are also rare in the literature. In this study, leptospires were detected in the pancreas at 12 hrs to day 3 p.i. by PCR and culture isolation from day 1 to day 3p.i. This also appears to be a rare finding in the literature.

Cardiac involvement in the pathogenesis of leptospirosis is seldom reported in the literature (Lourdault *et al.*, 2009). Merien *et al*., (1998) detected leptospires in the heart of guinea pigs infected with *leptospire icterohaemorrhagiae* at day 1 p.i. using immunofluorescence. This is in agreement with this study, in which leptospires were detected not only at day 1 p.i. but as early as 12hrs p.i., using culture isolation and PCR in this study.

Although, Lourdault *et al*., (2009) have used immunohistochemistry to detect leptospire in the adrenal gland of infected guinea pigs, but there is paucity of information in the literature on the isolation of leptospire in the adrenal gland using PCR and culture isolation. In this report, leptospire were recovered from the adrenal gland using PCR and culture isolation from 12 hrs to day 3 p.i.

Few studies have documented the demonstration of leptospire in the spleen of infected animals using different methods such as immunohistochemistry, immunofluorescence and quantitative PCR (Merien *et al*., 1998; Nally *et al*., 2004; and Lourdault *et al*., 2009). The findings in the spleen of the infected guinea pigs in this study are in agreement with these workers.

The recovery of leptospire from the liver and lung from 12 hrs to day 5 p.i. with the detection of leptospire in the kidney of infected guinea pigs throughout the entire period of this study was consistent with the information in the literature (Merien *et al*., 1998; Nally *et al*., 2004; and Lourdault *et al*., 2009).

In the literature, few reports are available demonstrating the pathogenesis of leptospirosis in different organs using EMJH medium and immunohistochemistry. Experimental infection of rats (Rattus norvegicus) with *L. interrogan Copenhageni* has been shown to be extensively disseminated to almost all organs during early phase of the disease. And this is followed by clearance of the organism within days in all these organs, except for selective survival and proliferation of leptospire in the kidney with subsequent leptospiruria (Athanazio *et al.,* 2008; Monahan *et al.,* 2008). In this study, all the organs examined such as liver, kidney, brain, spleen, pancreas, lung, heart, and adrenal gland were culturally positive to *leptospire interrogan* serovar *icterohaemorrhagiae* at 12 hrs and day 1 after infection.

The mode of leptospire dissemination and invasion within the host has been associated with genes encoding for various enzymatic secretions in the pathogenesis of leptospirosis which allow for degradation of the host cell membranes (Ren *et al.,* 2003). Among these are haemolysins and sphingomyelinase, which have been shown to initiates endothelial cells damage by increasing permeability, membrane aggregation, haemorrhages and localized ischemia and fusion (Goni and Alonso, 2002; Nascimento *et al*., 2004). This might have been responsible for the heamorrhages observed in this study, since the leptospiral antigens were demonstrated immunohistochemically in the capillary endothelial cells of the cerebral parenchyma in this study.

Little information is available in the literature on the clearance or removal of leptospiral organism from different organs. Monahan *et al.,* (2008) have affirmed the presence of circulating anti-leptospiral immunoglobulin (IgM and IgG) in rats at day 7 p.i. and while

Adler, *et al.,* (1980) detected the same in guinea pigs at day 3 p.i. In this investigation, the presence of immunohistochemically stained leptospire antigen in the cytoplasm of reticuloendothelial cells further gave evidence to the involvement of anti-leptospiral immunoglobulin before phagocytosis of leptospire organism in these organs.

Kidney has been regarded as the primary target organ of leptospire organism during both acute and chronic infection (Faine, 1999; Yang *et al*., 2001). But the clearance of the organism occurs within few days after infection in different organs, except for the selective survival and proliferation of leptospires in the kidneys (Athanazio, *et al*., 2008; Monahan *et al*., 2008). This assertion supported the argument that kidney is an immunopriviledge site and thus facilitate persistent colonization by leptospire.

Monahan *et al.,* (2009) stated that the mechanisms that facilitate the persistent colonization of leptospiral organism in the tubular lumina (immunopriviledge "site") are difficult to ascertain in the literature. In this study, the inability of leptospiral clearance from the kidney must have been as a result of the timing of the anti-leptospiral immunoglobulin production. Adler, *et al*., (1980) ascertained that antileptospiral immunoglobulin was decteted at day 3 p.i in their study using guinea pigs. In this present study, although anti-leptospiral immunoglobin was not determined, but leptospires were cultured and immunohistochemically detected in the kidney at 12hrs and day 1 p.i., before the production of anti-leptospiral immunoglobulin as affirmed by Adler, *et al.,* (1980). Thus, it is possible to deduce that leptospire might have "escape" into the tubular lumina (immunopriviledge site) before the commencement of antileptospiral immunoglobulin production in the kidney. Therefore, the assertion that kidney is the primary target organ by tropism might not be true, because leptospire initially disseminate to all tissues and not specifically targeting the kidney.

The hepatic histopathological changes observed in this study were consistent with those documented elsewhere (Nally *et al.,* 2004). The histopathological changes which consist of

focal hepatic necrosis, slight loss of hepatic cohesion and vacuolar degeneration are consistent with mild significant increase observed in the levels of the ALT from day's 3 to 7 p.i. in this study.

Ultrastructural studies carried out by Cheville *et al*., (1980) in swine demonstrated two phases of the infection in the kidney after heamatogenous route of entering. The first phase showed the migration of the organism through the endothelial cells into the interstitium (interstitial phase) which is usually characterized by oedema, vasculitis and leptospiremia. This is in agreement with this study, as leptospire antigens were observed immunohistochemically within the endothelial cells of the capillaries and the presence of oedema with heamorrhages at 12hrs, day 1 and day 3p.i. (fig.6.9.20). The second phase (tubular phase) during which leptospire associated with or move into the lumina of the proximal convoluted tubules (Cheville *et al*., 1980). In this study, leptospire were demonstrated immunohistochemically as early as day 1 p.i. migrating from interstitium into the epithelial cells of the proximal convoluted tubules (fig.6.9.24).

In rat's kidney, despite early migration and establishment of leptospire, morphological alteration in the kidney and specific lymphocyte infiltration were not observed until day 21 after infection (Nally *et al* ., 2005a; Tucunduva de Faira *et al*.,2007). In this investigation, tubular nephrosis and interstitial lymphocytic infiltration were observed as early as days 3 and 7 respectively. This suggests that leptospire enter the tubular lumina as early as day 1p.i. and before the commencement of the production of specific local immune response.

The pulmonary haemorrhagic phenomenon in acute leptospirosis has been documented in humans, dogs and guinea pigs (Gonçalves *et al.*, 1992; Pereira *et al*., 2002; Yang *et al.*, 2006). Deaths may occur in less than 3 days after the advent of respiratory signs and symptoms, which usually appear between the 4th and the 6th day of the disease (Pereira *et al.*, 2002). The histopathological changes observed in this study were consistent with the severe

pulmonary form of leptospirosis (SPFL) reported in humans (O'Neil *et al.*, 1992). A time-related severity of the pulmonary haemorrhages was observed by the increasing severity of the gross and histopathological changes.

Conflicting reports abound in the literature on the pathogenesis of thrombocytopenia observed in leptospire infected animals and human. Some investigators were of the opinion that thrombocytopenia observed in leptospirosis is as a result of disseminated intravascular coagulopathy (DIC) (Higgins and Couisineau 1977a, b; Higgins *et al*., 1980; da Silva *et al.*, 1995; Lomar *et al*., 2000), while some have contrary opinion (Nicodemo *et al.*, 1990; Nally *et al*., 2004; Yang, *et al.*, 2006). Various factors have been suggested by Nally *et al*., (2004) which might have been responsible for the disparity observed in the results of these investigators. These include different serovars of isolate used by these authors, the extent of in-vitro passage of the leptospire and infectious dose used in inoculation of the animals. In this study, fresh isolate of *leptospire icterohaemorrhagiae* was used and all the histopathological changes were in agreement with the report of those workers, who affirmed the involvement of DIC in the pathogenesis of thrombocytopenia observed in leptospirosis. This is evidenced by the presence of thrombi in the pulmonary vessels which attached to the mucosa surfaces and the immunohistochemical demonstration of the organism in the endothelial cells of the cerebral vessels. This suggests endothelial injury and consequently platelet aggregation and thrombi formation. Therefore, thrombi formation in this study might have likely been due to endothelial injury and platelet aggregation which consequently leads to thrombocytopenia. The immunoreactivity to the granular form of leptospire antigen in the cytoplasm of pulmonary alveolar macrophages in this study at day 3 p.i. is consistent with the findings of other workers (Nally, *et al*., 2004; Yang *et al*., 2006).

Leptospirosis has been shown to cause diffuse organ involvement due to extensive vasculitis (Levett, 2001; Lomar, 2002). Different investigators have documented the involvement of

pancreas in leptospirosis infection (Edward and Evarard, 1991; O'brien *et al.*, 1998; Casella and Scatena, 2000) in humans. In humans and animal model of leptospirosis, no gross observable pancreatic alterations have been reported (Arean, 1962), but in this study pin point or petechial haemorrhages were present on the serosal surfaces of the pancreas. Histopathologically, acute pancreatitis characterized by oedema, mild inflammatory lymphocytic infiltrates, haemorrhages, congestion and calcification have been consistent findings in human leptospirosis (Levett, 2001; Kaya *et al.*, 2005). In this study, histopathological changes such as oedema, congestion and haemorrhages were consistent with the report of these workers. However, fat necrosis and calcification observed in their reports were not observed.

Leptospiral encephalitis is one of the uncommon manifestations of leptospirosis in domestic animals (Cook *et al*., 2004). In humans, despite invasion of leptospire into the cerebrospinal fluid (CSF) in the early septicemic phase of the condition, neurological signs do not manifest until after the second week of illness. This has been attributed to antigen-antibody complex – induced inflammation (Levett, 2001; Panicker *et al.*, 2001). In this study, no neurological clinical manifestations were observed apart from depression in the first few days after infection. The gross morphological alteration of meningeal congestion is in agreement with works of other authors in humans (Area´n, 1962; Zaki and Spiegel, 1998; Panicker *et al.*, 2001) but there is a dearth of information on the cerebral histopathological changes associated with leptospiral infection in both domestic and experimental animals. In this study, lesions such as capillary endothelial cell damaged, mild to moderate perivascular oedema, perivascular cuffing, gliosis, neuronal degeneration and immunohistochemical reactivity of leptospiral antigens in the cerebral parenchyma, cytoplasm of glial cells, and damaged endothelial cells (fig.6.9.37-38, 40-41) were observed. These observations are rare findings in

the literature and appeared to be the first leptospiral cerebral histopathological changes in the guinea pigs model.

In humans, pathological findings in the heart include interstitial myocarditis with infiltration of predominantly lymphocytes and plasma cells, hemorrhages (particularly in the epicardium), mononuclear infiltration in the epicardium, pericardial effusions, and coronary arteritis have been documented in both human and few experimental studies (Lee, *et al.*, 1986; De Biase, *et al.*, 1987; de Brito, *et al*., 1987; Emmanouilides *et al*., 1994). This is in agreement with this study in which interstitial oedema and heamorrhages, myocardial degeneration and necrosis with non suppurative myocarditis were the consistent findings in this investigation. However, immunohistochemical staining of leptospire in the heart was not possible in this study.

The absence of immunohistochemical staining of leptospiral organisms in the pancreas, myocardial tissue and adrenal gland in this study might be due to low number of the organisms in these organs, or small area of the tissues were examined immunohistochemically as suggested by Ross *et al.*, (2011).

Summarily, the dog's leptospiral isolate used in this experimental model, mimic the clinico-haematological changes, dissemination kinectics and the gross and histopathological changes observed in the dogs in this study. This investigation unraveled the reason why leptospires were not detected from organs such as lung, pancreas, heart, spleen and the brain, except the kidney in the dogs, as leptospires were only present or detected for a few days (day 5 p.i.) in the organs of infected guinea pigs. It also showed that leptospiral organism escape into the tubular lumina as early day 1 p.i. which conferred the advantage of beign not cleared from the kidney as the antileptospiral antibodies might not have been produced at this time as suggested by Adler *et al.*, (1980).

7.0 CHAPTER SEVEN

7.1 CONCLUSION AND CONTRIBUTION TO KNOWLEGDE

This study has demonstrated that leptospirosis is one of the major causes of death in dogs in the South-western Nigeria, having 80 cases (76.9%) (43 cases from WSSS and 37 cases from EMJH medium) (Table 3.18) of leptospirosis out of 104 dogs examined. It also showed that the increasing trend of canine leptospirosis between 2003 to 2007 might have beeen due to different serovars in which dogs were not vaccinated against.

The high prevalence of leptospirosis in this study, especially in dogs cattle and wild rats suggest epidemiological significance of the disease in the study area. This might possibly leads to humans contacting the disease from these animals. The group of people that are likely to contact the disease are the abbatior workers, pet owners and the the clinicians.

The presence of *L. icterohaemohagiae* and *L. pomona* as the most most prevalent and significant serovars in wild rats and dogs suggest that wild rats are the source of infection in most cases of canine leptospirosis in the southwest zone of Nigeria.

Despite the fact that *L. icterohaemorrhagiae* and *L. pomona* were the most predominant serovars in this study, the presence of 3 unidentified serovars using monoclonal antibodies and lack of immunoreactivity by the WSss positive liver and kidneys samples in this study, suggest that there are other unidentified serovars in the dog population apart from grippotyphosa, pomona, canicola, icterohaemorrhagiae and bratislava isolated in this study. This might necessitate further investigations.

This study also showed that there have been changes in the leptospire serovars over the period of time from the traditionally known L.canicola and L. icterohaemorrhagiae to other serovars such as pomona, bratislava, gripptotyphosa and other unidentified serovars in this study. This suggest that the leptospire vaccine should be all encompassing by including all the identified serovars in this study in the vaccination programme of dogs to prevent the

disease in the country. It is also possible that the unidentified serovars in this study might have contributed to the high mortalities in dog populations over the years.

The gross picture of icterus and vascular disturbances of haemorrhages with the renal histopathological findings of interstitial nephritis and tubular nephrosis in this study were also consistent with works of other authors (Prescott, 2002). The presence of the same histological lesions in asymptomatic carriers such cattle and wild rats also showed that these animals demonstrate pathology in their kidneys, despite absence of visible clinical disease associated with renal failure. This study has also demonstrated that tubular colonization depends on the age or size of the animals as adult rats showed more tubular colonization than the juveniles. Concerning regional and tubular leptospiral localization and colonization, city rats used in this study seem to show more preference for cortico-medullary junction and distal convoluted tubules respectively. These finding are rare in the literatures and contribute to the existing knowledge of leptospirosis in the wild rats.

This investigation increases our understanding of potential routes of pathogenic leptospire transmission to other domestic animals and possibly humans especially serovars icterohaemorrhagiae, pomona, canicola and bratislava. This study also showed the possibility of domestic animals, especially dogs to be infected with leptospirosis when they are fed with infected and improperly cooked meat and milk.

Moreso, leptospiral antigens in the various organs such as pancreas, heart, lung, spleen, lymph nodes and brain of dogs were not detected. This might have probably been due to (1) clearance of leptospiral organism from these organs before death by the immune system or (2) due to the use of leptospirucidal antibiotics which might have cleared the organism from these organs before death as suggested by Ross *et al.*, (2011). But, the experimental aspect of this study have demonstrated that leptospires are quickly removed from different organs at

different times from 12hrs to day 5 p.i., except for the persistence of the organism in the renal tubules.

The dog's leptospiral isolate used in the experimental guinea pig model, mimic the clinico-haematological changes, dissemination kinectics and the gross and histopathological changes observed in the dogs. This work has demonstrated the need for the combination of different methodologies in the study of the pathogenesis or dissemination of leptospiral organism in an animal model. In this investigation, leptospire was not isolated beyond day 5 p.i. in the organs examined, apart from kidney. The inability to isolate leptospire from the organs (apart from kidney) of infected guinea pigs beyond day 5 p.i., confirm the reason why the organisms was not isolated from organs such as the brain, lung, spleen, pancreas, liver and the heart of infected dogs in this study. This is due to the clearance of the organism by the reticuloendothelal cells, since many of them were observed in the liver, lung and spleen immunohistochemically, demonstrating leptospire antigens in their cytoplasm. The "escape" of leptospiral organism from clearance from the kidney appears to be time dependent, in which leptospiral organism entered into the tubular lumina before the activation of antigen-antibody complex. This is demonstrated in this study, in which leptospires were present on the tubular wall as early as day 1 p.i. and the presence of mononuclear cells infiltration at day 7 p.i. These are rare findings in the literature and add to the existing knowledge on the pathogenesis of leptospirosis in guinea pigs model.

Although, leptospire has been detected in the adrenal gland of infected guinea pigs using immunohistochemistry (Lourdault *et al*., 2009), but there is a paucity of information in the literature on the isolation of leptospire in the adrenal gland and pancreas using PCR and culture isolation. In this report, leptospire were recovered from the adrenal gland and pancreas using PCR and culture isolation from 12 hrs to day 3 p.i. These are also rare

findings in the literature and add to the existing knowledge in the pathogenesis of leptospirosis.

Moreso, cultural isolation, PCR and immunohistochemistry detection of leptospiral antigens in the brain, with the associated cerebral histopathological changes in both domestic and experimental animals, to the best of the author's knowledge, are rare findings in the literature. This also complements existing knowledge, and adds new findings to the pathogenesis and pathology of leptospirosis in an experimental model.

These studies have contributed towards the understanding of the mechanisms of leptospire escape of the immune system through the use of WSSS and immunohistochemistry, its time dependent dissemination kinetics especially to the brain, pancreas and adrenal gland and induce pathology in these organs, which are rare findings in the literature.

The results presented in this investigation is in agreement with previous works on wild rats as the main reserviour host of different serovars of leptospires such as *L. Pomona, L. gripptotyphosa, L. bratislava, L. canicola*, and *L. icterohaemorrhagiae.*

Tubular nephrosis and lymphoplasmacytic interstitial nephritis were the most significant histopathological changes observed in leptospire positive kidney of rats which might not, in part be due to leptospirosis since the same lesions were observed in the leptospiral negative rats. Given the increase in observed carriage rate in rats in this investigations compared with earlier study in Nigeria (68% versus 4.5%), it is possible to suggest that this increase in prevalence rate might have high prevalence and transmission of leptospirosis to domestic animals, especially dogs within these period of study.

The study also demonstrated the usefulness of WSSS and immunohistochemistry in the determination of the duration of infection in the affected animals. The ability of these staining methods to localized leptospiral antigen at different stages of tissue invasion could be use to

determine whether infection is acute or chronic, especially in the kidney, as it is demonstrated in this study.

The culture, MAT, monoclonal antibodies, Warthin Starry silver stain and immunohistochemistry used in this study were in accordance with WHO guidelines (W.H.O., 2003) and are considered sufficient for the diagnosis and identification of reserviours host (Faine *et al.*, 1999). The methodologies used in this study are rapid and relatively inexpensive and provide an opportunity for the safe and specific detection of leptospires in tissue samples of animal origin. IHC and Warthin Starry silver stain can be used for the histopathological diagnosis of leptospirosis either as a primary approach or when the aetiology or the cause of death is uncertain because of unavailability of fresh samples where lives organism can be culture and isolated.

7.1. Further Studies

Consequent to the available information in this investigation, it has clearly shown that leptospirosis is an underreported disease in Nigeria. Thus, it is necessary that the following unexplored areas should be investigated.

All the livestocks including goat sheep, cat, local chicken, donkey, horse and other reserviour hosts with the occupational risk workers in the country should be investigated to know their state, since leptospirosis has been rarely documented in the subjects.

Isolation, characterization and sequencing of all the Nigeria isolates should be carried out since antigenically related serovars may be different genetically and the genetically similar serovars might be antigenically differs. Those that are not presently in the gene bank should be named accordingly and deposited there.

Further studies are also necessary in the development of all encompassing vaccines that will include all the serovars found in a particular species of animals .

7.2 Recommendations

This study has demonstrated that different serovars of leptospiral organisms abound in different reserviour hosts and there is a countinous leptospiral related-death of dogs and possibly humans with other domestic animals in the studied areas. The following specific actions are recommendations:

1) Concerted effort must be made to determine the prevalence rates of leptospirosis in humans, companion animals, livestock, and wildlife in Nigeria. This effort would include the identification of the serovars that occur in the various groups of animals, and which species serve as maintenance hosts for each serovar.
2) Field studies should be carried out in households within the cities where rodents occur in Nigeria to determine the primary habitats and mode of infection of dogs and other domestic animals.
3) Knowledge of the rodent populations is very important for effective and comprehensive rodent control program to keep the rate of their multiplication at bay. This program should be integrated with pest management strategy. Important components of this strategy would include sanitation and the rodent-proofing of buildings, routine rodent monitoring schedule, effective control methods, including traps and rodenticides, must be developed, made readily available and used. A corps of personnel trained in rodent biology, ecology, and management should be established and properly equipped.
4) Hygienically, the conditions of homes and the environment must be improved so that they are less favorable to the occurrence of leptospirosis. The presence of standing water in and around the dogs environment should be eliminated or minimized.
5) Considering the zoonotic importance of leptospirosis, a regular program of testing cattle at the abattoirs for leptospirosis should be initiated, with the subsequent removal of infected animals.

6) Vaccination of cattle should commence in earnest, since no vaccines are available presently for the immunization of cattle against leptospirosis in Nigeria.

7) Abattior workers (and others in occupations at risk) should be encouraged to use protective equipment and clothing and to practice good occupational hygiene. This effort should be incorporated into an expanded public education enlightenment program.

8) Dog's foods that involve incorporation of abattoir offals should be properly cooked and at no occasion should raw abattoir offals offered to dogs and cats to prevent leptospiral infection.

9) Presently, in Nigeria the report of occurrence of leptospirosis in human is scanty. This might possibly be due to lack of awareness of the occurrence of the disease in our environment. This call for effective leptospirosis educational campaign to reach all political, economic, cultural and social sectors and for the government to effect a leptospirosis surveillance and management program along with an effective rodent management program.

10) Lastly, all the aforementioned resolutions and recommendations might be a fruitless effort if the information and samples gathered from the field cannot be process and analyzed. This call for the establishment of leptospirosis laboratory in the country (presently, there is no laboratory where samples suspected of leptospirosis can be process) so that effective surveillance and diagnosis can be carried out.

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APPENDIXES

Appendix I

Warthin Starry silver stain procedure

For the silver staining procedure, Young's modification of Warthin and Starry (1920) was used in identification of the Leptospires organism in the tubular lumina of the rats. Briefly, sections were dewax, rinse in alchol and deionized or distill water and latter rinse with buffer solution (1.5ml of 0.2m sodium acetate, 18.5ml of 0.2m acetic acid and 480ml of de-ionised water). The sections were then placed in a pre-heated solution A (1% Silver nitrate) at 56^0c in water bath for 1 hour. Five minutes before the hour was up, 2.5ml of solution C (2% Silver nitrate) was added to 45ml of solution B (3.1g gelatine in 45ml buffer, pH 3.7.). When the hour was up, solution A was tipped out, leaving the slides in the staining jar and 3ml of solution D (4% Hydroquinone solution: 0.4g hydroquinone in 10ml buffer, pH 3.7) was quickly added to the mixtures of silver/ gelatine and pour into the staining jar with the slides. The slides were treated in the mixture until they were golden brown in color (1-3 minutes). The slides were then carefully washed in warm tap water, dehydrated, cleared and mounted.

Appendenxi 2

Group Statistics (PCV)

	V1	N	Mean	Std. Deviation	Std. Error Mean
12hrs	infected	3	34.33	2.309	1.333
	control	2	41.50	2.121	1.500
24hrs	infected	3	38.33	2.887	1.667
	control	2	44.00	.000	.000
48hrs	infected	3	36.00	4.359	2.517
	control	2	41.50	2.121	1.500
72hrs	infected	3	33.33	2.887	1.667
	control	2	42.50	.707	.500
96hrs	infected	3	28.33	8.327	4.807
	control	2	28.00	2.828	2.000
120hrs	infected	3	28.33	3.786	2.186
	control	2	39.00	1.414	1.000

Appendenxi 3

Group Statistics (RBC)

	V1	N	Mean	Std. Deviation	Std. Error Mean
12hrs	infected	3	3.667	.2517	.1453
	control	2	4.300	1.1314	.8000
24hrs	infected	3	3.533	.2082	.1202
	control	2	4.000	.0000	.0000
48hrs	infected	3	3.300	.5568	.3215
	control	2	3.250	.3536	.2500
72hrs	infected	3	4.133	.2517	.1453
	control	2	4.000	.7071	.5000
96hrs	infected	3	3.900	.3000	.1732
	control	2	5.000	.2828	.2000
120hrs	infected	3	3.967	.7095	.4096
	control	2	5.150	.3536	.2500

Appendenxi 4

Group Statistics (Hb)

	Hb (g/dl)	N	Mean	Std. Deviation	Std. Error Mean
12hrs	infected	3	13.267	1.4048	.8110
	control	2	16.300	.2828	.2000
24hrs	infected	3	14.633	.7506	.4333
	control	2	14.800	.0000	.0000
48hrs	infected	3	14.333	1.1547	.6667
	control	2	14.850	.4950	.3500
72hrs	infected	3	11.433	1.2423	.7172
	control	2	14.300	.9899	.7000
96hrs	infected	3	9.833	4.9652	2.8667
	control	2	9.250	1.7678	1.2500
120hrs	infected	3	7.967	2.9023	1.6756
	control	2	13.950	.2121	.1500

Appendenxi 5

Group Statistics (MCV)

	MCV(fl)	N	Mean	Std. Deviation	Std. Error Mean
	infected	3	94.200	12.8515	7.4198
	control	2	99.300	21.2132	15.0000
24hrs	infected	3	108.433	2.5166	1.4530
	control	2	110.000	.0000	.0000
48hrs	infected	3	111.067	21.2890	12.2912
	control	2	128.800	20.5061	14.5000
72hrs	infected	3	81.100	11.3767	6.5684
	control	2	107.800	17.2534	12.2000
96hrs	infected	3	73.967	26.4379	15.2639
	control	2	55.950	2.4749	1.7500
120hrs	infected	3	73.233	18.3587	10.5994
	control	2	75.850	2.4749	1.7500

Appendenxi 6

Group Statistics (MCHC)

	MCHC(g/dl)	N	Mean	Std. Deviation	Std. Error Mean
12hrs	infected	3	38.633	2.5146	1.4518
	control	2	39.350	2.7577	1.9500
24hrs	infected	3	38.233	1.6166	.9333
	control	2	33.600	.0000	.0000
48hrs	infected	3	40.033	3.7899	2.1881
	control	2	35.800	.7071	.5000
72hrs	infected	3	34.267	.8505	.4910
	control	2	33.650	2.8991	2.0500
96hrs	infected	3	32.933	9.0908	5.2486
	control	2	32.900	2.9698	2.1000
120hrs	infected	3	28.633	10.9098	6.2988
	control	2	35.800	.7071	.5000

Appendenxi 7

Group Statistics (Reticulocytes)

	Reticulocyte	N	Mean	Std. Deviation	Std. Error Mean
12hrs	infected	3	.000	.0000	.0000
	control	2	3.450	2.3335	1.6500
24hrs	infected	3	7.333	7.9952	4.6160
	control	2	3.000	1.4142	1.0000
48hrs	infected	3	7.867	7.8009	4.5038
	control	2	5.500	2.8284	2.0000
72hrs	infected	3	7.167	7.2072	4.1611
	control	2	17.800	22.6274	16.0000
96hrs	infected	3	3.800	1.6523	.9539
	control	2	12.400	2.8284	2.0000
120hrs	infected	3	2.067	2.3352	1.3482
	control	2	2.600	.1414	.1000

Appendenxi 8

Group Statistics (in vivo MDA)

	In vivo MDA content(106 µM/ml)	N	Mean	Std. Deviation	Std. Error Mean
12hrs	infected	3	.3800	.17776	.10263
	control	2	.4150	.51619	.36500
24hrs	infected	3	.2933	.23116	.13346
	control	2	.4000	.00000	.00000
48hrs	infected	3	1.9533	3.09926	1.78936
	control	2	.3750	.47376	.33500
72hrs	infected	3	.4500	.13229	.07638
	control	2	.2200	.24042	.17000
96hrs	infected	3	.5067	.16166	.09333
	control	2	.5600	.21213	.15000
120hrs	infected	3	.3967	.21779	.12574
	control	2	.5150	.27577	.19500

Appendenxi 9

Group Statistics (in vitro lipid peroxidation)

	In vitro MDA content(106 µM/ml)	N	Mean	Std. Deviation	Std. Error Mean
12hrs	infected	3	1.7200	.35511	.20502
	control	2	.5600	.04243	.03000
24hrs	infected	3	2.9567	.57709	.33318
	control	2	.0750	.03536	.02500
48hrs	infected	3	3.5167	1.58052	.91251
	control	2	.0550	.00707	.00500
72hrs	infected	3	4.7233	2.29269	1.32369
	control	2	.3350	.00707	.00500
96hrs	infected	3	3.6233	1.26469	.73017
	control	2	1.2650	1.15258	.81500
120hrs	infected	3	3.6567	1.50114	.86669
	control	2	.9250	.00707	.00500