CHAPTER 1

1. INTRODUCTION

Canine leptospirosis is a bacterial zoonosis with worldwide distribution causing septicaemia, renal and hepatic disease, coagulopathies and other abnormalities, with a case fatality rate of 10–20% in dogs (Cullen *et al.*, 2004). Leptospirosis is caused by pathogenic spirochaetes once classified as a single species *Leptospira interrogans*, now subdivided into at least 16 genomospecies (Adler and Moctezuma, 2010). Within the leptospires, based on antigenic differences, there are at least 23 different serogroups and 218 serovars, which do not reliably relate to the causative genomospecies (Plesko and Hlavata, 1971). The infected dogs may be carriers of leptospiral organisms for 1-2 years in a sub-clinical form or with presentations of renal failure. Within this period, the dogs are very important hazardous carriers for susceptible human and animal hosts.

Leptospires are maintained by a wide range of reservoir hosts such as rats and domestic animals such as cattle, pigs and historically, dogs. Reservoir hosts typically carry and transmit specific host-adapted serovar(s). Transmission generally occurs after a susceptible animal is directly exposed to leptospires in an infected host's urine or contaminated water, mud, or moist soil (Bulach *et al.*, 2006). In their reservoir host, leptospires escape the immune system in the proximal convoluted renal tubules allowing the infected animal to become a persistent shedder (Levett, 2001). Following shedding in urine, these bacteria may survive for some months under appropriate moist or wet environments; however, survival is very poor in dry or cold environments (Adler and Moctezuma, 2010).

Shedding animals pose a public health risk and leptospires can be transmitted to humans through contact with urine-contaminated environments, particularly water (Sykes *et al.*,

2011). Human leptospirosis is usually associated with direct contact with shedding companion animals, occupational exposure (sewage and agricultural workers, and veterinarians) or outdoor recreational activities such as swimming, boating, or endurance competitions (Levett, 2001).

Canine leptospirosis has been reported from different parts of the world including Brazil (Rubel *et al.,* 1997), Europe (Ellis, 2010), Philippines (Weekes *et al.,* 1997), South Africa (Roach *et al*., 2010), and USA (Gese *et al.,* 1997).

Historically, the serovars L. Canicola and L. Icterohaemorrhagiae have been of major concern for dogs in Zimbabwe. Consequently, dogs are currently vaccinated against leptospirosis using a killed bacterin containing these two serovars (Levett, 2001). However no studies have been done to ascertain the prevalence of these and the presence of other serovars. Since the re-emergency of leptospirosis in North American countries, the other two most commonly reported wildlife-associated serovars have been L. Grippotyphosa and L. Pomona (Levett, 2001). This prompted an introduction in 2001 of a canine vaccine, which contains serovars L. Grippotyphosa and L. Pomona in addition to L. Canicola and L. Icterohaemorrhagiae (Bulach *et al*., 2006). This vaccine is however not yet available for use in Zimbabwe.

It has been postulated that the re-emergence of leptospirosis and changes in the epidemiology of the infecting serovars may be due to urbanization of rural areas in the 1980s and 1990s, which provided greater opportunity for contact between animals and wildlife reservoirs (Murray *et al*., 2009b). Investigations of the resurgence of canine leptospirosis revealed conflicting findings for risk factors such as age, sex, and breed of dogs. However, environmental risk factors such as increased precipitation, warmer temperatures, and

seasonality of cases have been fairly consistently identified (Jost *et al*., 1989). In Zimbabwe, although leptospirosis due to L. Canicola and L. icterohaemorrhagiae have been reported in other animals (Dalu and Feresu, 1997), detailed studies of the prevalence of these and other serovars in dogs and risk factors for their occurrence have not been studied in detail. Due to no cross protection among the different serovars and reports of other new serovars infecting dogs, conducting a sero-survey of canine leptospirosis in urban and rural areas of the country was warranted. Furthermore, in spite of the zoonotic importance of the different *Leptospira* serovars, studies on public awareness of leptospirosis have not been conducted. Therefore, this study was conducted to determine the seroprevalence and risk factors associated with canine leptospirosis and, public awareness of the disease.

1.1 Specific Objectives

1.1.1 To determine the seroprevalence of leptospirosis due to four *L. interrogans* serovars in dogs from selected areas of Zimbabwe.

1.1.2 To determine the association between canine leptospirosis seropositivity and the clinicopathological features suggestive of leptospirosis, location and sex.

1.1.3 To assess the public awareness of the disease in urban areas around Harare.

CHAPTER II

2. REVIEW OF LITERATURE

2.1 Aetiology

Leptospires are spirochetes, about 0.1m m in diameter and 6–20mm in length that include both saprophytic and pathogenic species comprising the genus *Leptospira,* which belongs to the family Leptospiraceae, order Spirochaetales (Faine *et al*., 1999). Recently, the status of species has been given to the previously described genomospecies 1, 3, 4 and 5, resulting in a family comprising of 13 pathogenic *Leptospira* species (*L. alexanderi, L. alstonii* (genomospecies 1), *L.borgpetersenii, L. inadai, L. interrogans, L. fainei, L. kirschneri, L. licerasiae, L. noguchi, L. santarosai, L. terpstrae* (genomospecies 3), *L. weilii, L. wolffii,* with more than 260 serovars (Faine *et al*., 1999). Saprophytic species of *Leptospira* include *L. biflexa, L. meyeri, L. yanagawae* (genomospecies 5), *L. kmetyi, L. vanthielii* (genomospecies 4), and *L. wolbachii,* and contain more than 60 serovars. The serovar classification of *Leptospira* is based on the expression of surface-exposed epitopes in a mosaic of the lipopolysaccharide (LPS) antigens, while the specificity of epitopes depends on their sugar composition and orientation (Faine *et al*., 1999).

Leptospires have distinctive hooked ends. The two periplasmic flagella (FlaA and FlaB) with polar insertions are located in the periplasmic space and are responsible for motility. The FlaA and FlaB proteins constitute the flagellar sheath and core, respectively. However, a FlaB mutant has been shown by electron microscopy to be non-motile and deficient in endo-flagella (Picardeau *et al*., 2001).

Leptospires have been shown to have a typical double membrane structure in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and overlaid by an outer membrane (Cullen *et al*., 2004). Within the outer membrane, the LPS constitute the main antigen for *Leptospira* and it is structurally and immunologically similar to the LPS from Gram negative organisms. However, it is relatively non-toxic to cells or animals and is 12 times less lethal for mice when compared with *E. coli* LPS (Faine *et al*., 1999). Leptospiral Lipid A contains some unusual features (Que-Gewirth *et al*, 2004) that include a modified glucosamine disaccharide unit which is phosphorylated and methylated. In addition to LPS, structural and functional proteins, which are mainly lipoproteins, form part of the leptospiral outer membrane (Cullen *et al*., 2005). Integral membrane proteins such as the porin OmpL1 (Shang *et al*., 1995) and the type two secretion system (T2SS) secretin GspD (Rodrı´guez Reyes *et al*., 2005), are also located in the outer membrane of *Leptospira* and have been shown to be antigenic.

Leptospires are obligate aerobes with an optimum growth temperature of 28–30°C (Adler and Moctezuma, 2010). They grow in simple media enriched with vitamins B1 and B12, long-chain fatty acids, and ammonium salts. Long-chain fatty acids are utilized as the sole carbon source and are metabolized by b-oxidation (Faine *et al*., 1999).

Growth of leptospires in media containing serum or albumin and in protein-free synthetic media has been described. Several liquid media enriched with rabbit serum have been used in the past (Faine *et al*., 1999). Currently, the most widely used medium is Ellinghausen-McCullough-Johnson-Harris (EMJH) and is based on the oleic acid, bovine serum albumin and polysorbate (Tween) medium. Some strains require the addition of pyruvate or rabbit serum for initial isolation. Growth of contaminants from clinical specimens can be inhibited

by the addition of 5-fluorouracil, gentamiycin, nalidixic acid or rifampicin (Faine *et al.*, 1999). Growth of leptospires is often slow on primary isolation, and cultures have to be retained for about 13 weeks before being discarded. Agar may be added at low concentrations (0.1–0.2%).

In such 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 and is known as a Dinger's ring or disk (Adler and Moctezuma, 2010). Leptospiral cultures are maintained by repeated subculture or by storage in semisolid agar containing haemoglobin. Long-term storage in liquid nitrogen also yields good results and is the preferred method of storage for maintaining virulence.

2.2 Genomics and molecular biology

The slow growth of leptospires in liquid and solid media and their relative metabolic inertness in phenotypic tests usually used for other bacteria indicates that they have few physiological or colonial markers. Mutants have been selected to grow in media containing boiled as opposed to fresh serum. There are no studies of the genetic basis of these mutations and the strain variants that usually form small colonies appearing spontaneously in some strains (Picardeau *et al.*, 2008).

Virulence is lost on subculture in the laboratory, but the small proportion of virulent leptospires can usually be selected from the culture population by inoculating experimental animals. Naturally occurring plasmids have not been detected in *Leptospira* and the mechanisms of gene transfer that are assumed to occur between leptospires have not been elucidated (Wang *et al.*, 2007). Transformation in nature has not been reported among

leptospires. A bacteriophage for *L. biflexa* was described, but none has been found that affects any of the pathogenic species. However, this key discovery formed the basis of the only plasmid shuttle vector developed between *E. coli* and *L. biflexa* (Saint Girons *et al*., 2000) but it does not replicate in pathogenic *Leptospira* spp.

Depending on species, the chromosome of *Leptospira* is characterized by a G + C content of 35–41 mol%, with a genome size of 3.9–4.6 Mb. There are currently six published leptospiral genome sequences: two serovars (L. Lai and L. Copenhageni) of *L. interrogans*, two strains of *L. borgpetersenii* serovar L. Hardjo and two strains of *L. biflexa* serovar L. Patoc (Bulach *et al*., 2006; Nascimento *et al*., 2004; Picardeau *et al*., 2008; Ren *et al*., 2003). Both *L interrogans* and *L. borgpetersenii* have two circular chromosomes and their genomes are characterized by a high degree of genome plasticity, with evidence of large-scale genomic rearrangement. The most striking difference between *L. interrogans* and *L. borgpetersenii* is the much larger number of pseudogenes, insertion sequences and remnants in the latter. Limited regions of synteny are shared between their large chromosomes, probably as a result of recombination events between insertion sequences (IS).

The *L.borgpetersenii* genome is approximately 700 kb smaller and has a lower coding density than *L. interrogans*, suggestive of decay through a process of IS-mediated reduction of the functional genome. Additionally, many genes which are intact in *L. interrogans* have accumulated disruptive point mutations in *L. borgpetersenii* (Bulach *et al*., 2006). The significant loss of gene function is not random, but is centred on impairment of environmental sensing and metabolite transport and utilization. These features distinguish *L. borgpetersenii* from *L. interrogans*, a species with minimal genetic decay, and which survives extended passage in aquatic environments encountered by mammalian hosts. On the

other hand, *L. borgpetersenii* survives poorly in the environment and it was concluded that it is in the process of evolution towards dependence on a strict host-to-host transmission cycle (Bulach *et al*., 2006).

The saprophytic *L. biflexa* possesses a third circular replicon of 74 kb, designated p74, not present in pathogenic species (Picardeau, 2008). The presence of housekeeping genes on p74 which have orthologs located on the large chromosome in pathogenic *Leptospira* suggests that p74 is essential for the survival of *L. biflexa*. For example, recBCD are located on the large chromosome in *L. interrogans* and *L. borgpetersenii*, but are located on p74 in *L. biflexa* (Wang *et al*., 2007). Mutation of these housekeeping genes in other bacterial species can affect transformation competence and viability. Comparative genomics of the two pathogenic and one saprophytic species has identified 2052 genes common to all; the core leptospiral genome findings consistent with a common origin for leptospiral saprophytes and pathogens (Adler and Moctezuma, 2010). Genome comparisons also allow the identification of pathogen-specific genes. The overall proportion of genes encoding proteins of unknown functions is 40% (Bulach *et al*., 2006). However, genes of unknown functions are over represented in the genes unique to pathogenic species. Of 627 genes unique to *L. interrogans*, more than 500 (80%) encode hypothetical proteins (Picardeau, 2008). For *L. borgpetersenii* more than 200 out of 265 (75%) unique genes encode proteins of unknown function (Wang *et al*., 2007). These data are consistent with the notion that *Leptospira* possesses unique virulence factors which cannot be identified in other bacteria.

2.3 Epidemiology

Leptospirosis occurs worldwide; however, it is common to find endemic disease in a particular geographic region caused by infections with only one, or several, serovars. Despite being adapted to "primary reservoirs hosts"; which are commonly wildlife species *Leptospira* species also occur in almost any other mammalian hosts as "incidental or accidental hosts". The dog is the "primary reservoir host" to L. Canicola and L. Bataviae. Incidental hosts for L. Canicola include rats, raccoons, hedgehogs, voles and skunks while those for L. Bataviae are hedgehogs and voles. Dogs may also become infected with several other serovars and serve as "accidental or incidental hosts". Historically, L. Canicola and L. Icterohaemorrhagiae serovars have been associated with clinical disease in the dog. The primary reservoir host for L. Icterohaemorrhagiae is the rat and its incidental hosts are mice, raccoon, opossum, hedgehog, fox, woodchuck, skunks, and muskrats. However, the disease picture is reported to have changed in the North-eastern USA where several hundred cases of leptospirosis were reported on Long Island, New York (USA) in 1996. Since that time, both L. Grippotyphosa and L. Pomona have become more prevalent in that region. (The primary reservoir host for L. Grippotyphosa is the vole and its incidental hosts are mice, rats, raccoons, opossums, foxes, squirrels, skunks, hedgehogs, muskrats and moles. For L. Pomona the primary reservoir hosts are the cow and pig and the incidental hosts are deer, mice, raccoon, opossum, hedgehog, fox, woodchuck and the vole. L. Bratislava, whose primary reservoirs are pigs and horses, emerged in 2000 as an additional problem (Adler and Moctezuma, 2010).

Many leptospiral infections are asymptomatic with some cases being undiagnosed and hence, the reported prevalence/incidence of leptospirosis in dogs may be underestimated In addition,

leptospirosis is usually not included in the differential diagnosis of acute renal disease by many veterinarians. Furthermore, sero-conversion does not always correlate with overt clinical disease in the dog.

Leptospires do not multiply outside of the host animal species, but they survive well in the environment under optimal conditions (Sykes *et al*., 2011). In order for direct infection to occur, dogs should be exposed to leptospires from infectious urine, via transplacental and venereal routes, bite wounds, or ingestion of contaminated meat. The most common source of leptospirosis in dogs is contaminated water. Indirect transmission also occurs from vegetation, soil, or food contaminated by infectious urine (Bulach *et al*., 2006)

Pathogenic leptospires infect a wide range of domestic and wild animals and are shed from the renal tubules. Naive animals become infected via intact mucous membranes or abraded skin through contact with infected urine or urine-contaminated soil, water, food, or bedding. Leptospires do not replicate outside of the host but may remain viable for weeks to months in soil saturated with urine (Picardeau *et al*., 2008).

Transmission has also occurred after bite wound inoculation, ingestion of infected tissues venereal and placental transfer. In a study from Thailand, ingestion of raw meat by dogs was associated with leptospirosis (Wang *et al*., 2007). The organism prefers temperatures around 30°C while freezing and UV radiation inactivate leptospires. Contact with slow-moving or stagnant warm water is a risk factor for humans (Duncan *et al*., 2012). Outbreaks of the disease in dogs have followed periods of higher rainfall, with overall peak seasonal distributions occurring in the late fall in the United States (Sykes *et al*., 2011). The time of year for peak leptospirosis incidence varies geographically depending on local rainfall patterns and periods of freezing temperatures. In some geographic regions, disease

generally occurs in dogs that are exposed to or drink from rivers, lakes, or streams, or dogs roaming on rural properties (Bulach *et al*., 2006).

Suburban backyard dogs may become exposed after contact with urbanized wild animal populations. In developing countries, access to sewage increases risk of the disease in dogs (Sykes *et al*., 2011). Outdoor, intact male working dogs have been shown to be at higher risk, although dogs of any age, breed, and sex may become infected (Miller *et al*., 2011). In areas where wild animal species access suburban backyards, small breed dogs with minimal contact with water sources may be at risk. Contact with rodents also may pose a risk to these dogs, as well as dogs residing within cities (Duncan *et al*., 2012).

2.4 Pathogenesis

Leptospires enter the body through small cuts or abrasions of mucous membranes such as the conjunctiva or through wet skin. They circulate in the blood stream, with a bacteraemia phase lasting for up to 7 days. After reaching peak levels in blood and tissues, lesions due to the action of undefined leptospiral toxin(s) or toxic cellular components and consequent symptoms appear (De Brito *et al*., 1992). The primary lesion is damage to the endothelium of small blood vessels leading to localised ischemia in organs, resulting in renal tubular necrosis, hepatocellular and pulmonary damage, meningitis, myositis and placentitis (De Brito *et al*., 1992). Haemorrhages occur in severe cases as do jaundice, and frequently, platelet deficiency. There is usually a mild granulocytosis and splenomegaly. Once circulating antibodies appear, leptospires are removed from the circulation and tissues by opsono-phagocytosis (Merien *et al*., 1997). Tissue damage, even though it is severe, may be reversible and followed by complete repair (e.g. kidney, liver) although long lasting damage (e.g. myocarditis) may be a complication and may lead to scarring, well recognised in the

kidneys of pigs and dogs, where it may be observed macroscopically as ‘‘white spots’’ (Merien *et al*., 1997).

The mechanisms by which leptospires cause host tissue damage and disease are not well defined. In particular, the molecular basis for virulence remains unknown, due mainly to the absence, until recently, of genetic tools for the manipulation of *Leptospira*. There have been reports of the pathogenic mechanisms, but in almost all cases the specific leptospiral component responsible for the activity investigated was not identified. For example, virulent leptospires were shown to adhere to cultured renal tubular epithelial cells (Ballard *et al*., 1986), but the leptospiral adhesin was not identified. Virulent leptospires are resistant to complement and to killing by neutrophils in non-immune hosts, but are rapidly killed by either mechanism in the presence of specific antibody. There is no unequivocal evidence for a classical secreted toxin in *Leptospira* infection. However, in the kidneys of experimentally infected guinea pigs, endothelial damage was closely associated with the presence of remnants of leptospiral cells (De Brito *et al*., 1992). The ability of leptospires to invade Vero cells and to induce apoptosis in macrophages was correlated with virulence (Merien *et al*., 1997). Significantly, both properties were lost very rapidly upon *in vitro* subculture. A 36 kDa fibronectin binding protein was identified in a virulent strain of L. Icterohaemorrhagiae but was absent in an avirulent variant and in a saprophyte strain (Merien *et al*., 2000); but the variant was not genetically defined. Leptospiral LPS resembles standard Gram negative LPS chemically and immunologically. However, it is substantially less active in standard tests for endotoxin activity, such as rabbit pathogenicity, mouse lethality, Schwartzman reaction and B-cell mitogenicity. Whether this reduced biological activity is due to unique properties of leptospiral Lipid A (Que-Gewirth *et al*., 2004; Werts *et al*., 2001) remains unknown.

Hemolysins have been reported in a number of leptospiral serovars. Not surprisingly therefore, a range of genes encoding sphingomyelinases which are either cell-associated and/or extracellular have been identified in different *Leptospira* species; at least seven SphA-like genes were detected among the pathogenic *Leptospira*, including a postulated pore-forming haemolysin SphH (Picardeau *et al*., 2008). The significance in pathogenesis of the other Sph sphingomyelinases has not been determined experimentally. However, the recent availability of genome sequences from pathogenic and saprophytic *Leptospira* spp. (Bulach *et al*., 2006; Nascimento *et al*., 2004; Picardeau *et al*., 2008; Ren *et al*., 2003) coupled with the recent development of mutagenesis systems (Bourhy *et al*., 2005; Murray *et al*.,2008) has allowed a more detailed and genetically defined investigation of cellular and molecular pathogenic mechanisms in leptospirosis. For example, the absence of any sphingomyelinase genes in the saprophyte L. Biflexa (Bulach *et al*., 2006) argues for a role in pathogenesis for these enzymes rather than in environmental scavenging of nutrients.

The first genetically defined virulence factor in *Leptospira* was the OmpA-like, surface lipoprotein Loa22 (Ristow *et al*., 2007). A transposon mutant of loa22 was attenuated in both hamster and guinea pig models of acute leptospirosis. The function of Loa22 remains unknown and it is noteworthy that a Loa22 homologue is found in *L. biflexa*. Recently, the gene hemO, encoding a haeme oxygenase (Murray *et al*., 2008) was shown to be involved in, but not essential for, virulence in hamsters (Murray *et al*., 2009a).

A set of six surface proteins, termed LenABCDEF, possess structural and functional similarity to mammalian endostatins and were shown to bind both the complement regulator factor H and host lamina (Stevenson *et al*., 2007). Interestingly, all except LenA also bound fibronectin. The significance and role of these activities in pathogenesis remain undefined.

The major surface protein LipL32 has likewise been shown to bind host lamina (Hoke *et al.*, 2008), collagen and fibronectin (Hauk *et al.*, 2008). LipL32 is unique to and highly conserved across pathogenic *Leptospira* spp. It is therefore surprising that it is not involved in either acute leptospirosis in hamsters nor in colonisation of rat kidneys (Murray *et al.*, 2009b). A similar finding seems to apply to the surface exposed Lig proteins; since they are present only in pathogenic species and their expression was lost upon laboratory subculture with a concomitant loss of virulence (Matsunaga *et al.*, 2003). Both LigA and LigB bind fibronectin and their expression is up regulated under conditions of physiological osmolarity (Choy *et al.*, 2007). However, inactivation of LigB did not affect virulence for hamsters (Croda *et al.*, 2008). The results of the above studies all indicate a high degree of redundancy in leptospiral proteins involved in adhesion, survival *in vivo* and renal colonisation and this suggested that it will be difficult to identify and define virulence factors with single gene inactivation.

Clinical forms of the disease are influenced by several factors, including the host, which may either be a primary reservoir host or an incidental host. The disease in primary reservoir hosts tends to be more chronic, or asymptomatic with weak antibody responses (Croda *et al.*, 2008). In contrast, the disease in an incidental host tends to be acute and severe with marked antibody responses. The spectrum of disease in the dog ranges from subclinical, to subacute, acute (severe), or chronic; there may also be abortions with or without placentitis (Croda *et al.*, 2008).

Initially, leptospires penetrate the mucus membranes or intact or abraded skin. Then, over the next 4 to 11 days, organisms rapidly invade the bloodstream, creating a leptospiremia (Choy *et al.*, 2007). Early leptospiremia is associated with the clinical signs of fever, transitory

anaemia due to haemolysis, leukocytosis, haemoglobinuria and albuminuria (Choy *et al*., 2007). In susceptible dogs, leptospires usually establish a septicaemia and spread systemically to the internal organs, including the liver and kidneys, or to the placenta and foetus.

The extent of development of specific lesions depends on the particular serovar and its virulence, as well as the dog's immune status. If a dog had been vaccinated, it still may have antibodies in its serum, or it may mount an anamnestic response in the absence of antibodies (Hoke *et al*., 2008).

The described virulence factors of *Leptospira* include adherence factors associated with outer surface proteins (OSP) which allow attachment to host fibronectin and collagen, as well as unknown factors which allow invasion across mucus membranes or moist, softened skin (Hoke *et al*., 2008). Additional factors include the endotoxic activity of *Leptospira* lipooligosaccharide (LOS) and its action on monocytes; release of lymphokines, eliciting of disseminated intravascular coagulation (DIC) reactions. Haemorrhage and bleeding abnormalities, thrombocytopenia and platelet aggregation also result due to release of lymphokines. L. Icterohaemorrhagiae usually causes fever, haemorrhage, anaemia, and jaundice; whereas severe acute kidney failure and/or chronic active hepatitis is common with L. Grippotyphosa, resulting in a more severe disease than that caused by L. Pomona (Hauk *et al*., 2008). Infections with L. Pomona are often subclinical, but a chronic carrier state is common. Infection of dogs with the host adapted L. Canicola commonly results in chronic interstitial nephritis (Hauk *et al*., 2008).

Young unvaccinated dogs, or whose dams were not vaccinated, are at a greater risk of severe disease and death that may occur due to an acute septicaemia or haemolytic anaemia. Previously vaccinated older dogs that, later, become naturally infected with a field strain homologous to a vaccine serovar generally have minimal clinical signs (Hoke *et al*., 2008).

During the period of tissue invasion there may be liver necrosis as well as capillary and endothelial cell damage. As a consequence, petechial haemorrhages may occur in the renal parenchyma together with vascular damage, focal interstitial nephritis, anaemic anoxia, and haemoglobinuric nephrosis (Hoke *et al*., 2008). At this stage death may occur due to renal failure caused by interstitial nephritis.

Towards the end of the bacteraemia stage, 7 - 10 days post infection, the fever usually subsides and leptospires are cleared from the bloodstream as antibodies emerge (Croda *et al*., 2008). Recovery occurs as antibodies increase in the blood and the bacteraemia ends; the rapidity of recovery depends on the degree of organ damage (Croda *et al*., 2008). Leptospires which have localized in the kidney tubules, the eye, or the reproductive tract are sheltered from the bactericidal effects of serum antibody; a persistent leptospiruria may therefore develop, with periodic episodes of fever.

Urine shedding may last for prolonged periods, but antibody levels eventually decline since the leptospires, protected in the renal tubules, do not stimulate antibody production (Hoke *et al*., 2008). Eventually, recovered but shedding dogs may be seronegative when tested; however, the organisms continue to multiply and persist (Croda *et al*., 2008).

A detailed knowledge of the biology of *Leptospira* and the pathogenesis of leptospirosis lags behind that now elucidated for many bacterial infections. The main reason has been the lack

of standard genetic tools for mutagenesis, transformation and complementation that have been available for other bacterial pathogens for up to 25 years. Comparative genomics analyses suggest that *Leptospira* may possess unique virulence factors. However, with the recent development of transposon and targeted mutagenesis systems, it is anticipated that the next few years will bring major advances in the understanding of how pathogenic *Leptospira* spp. interact with the environment and with their mammalian hosts to cause disease.

2.5 Leptospirosis: The disease

Leptospirosis is a systemic disease of humans and domestic animals, mainly dogs, cattle and swine, characterized by fever, renal and hepatic insufficiency, pulmonary manifestations and reproductive failure. Clinical signs are quite variable; most cases are probably inapparent and associated with host-adapted serovars such as L. Canicola in dogs, L. Bratislava in horses and pigs, L. Hardjo in cattle and L. Australis and L. Pomona in pigs (Andre´Fontaine, 2006; Bernard, 1993; Ellis *et al*., 1986; Grooms, 2006). However, other serovars can be involved in more serious disease. Four syndromes have been identified in dogs: icteric, haemorrhagic, uremic (Stuttgart disease) and reproductive (abortion and premature or weak pups) (Grooms, 2006). Typical leptospirosis in dogs may present with fever, jaundice, vomiting, diarrhoea, intravascular disseminated coagulation, uremia caused by renal failure, haemorrhages and death (Bolin, 1996).

A chronic manifestation of leptospirosis is commonly seen in horses as recurrent uveitis (Rohrbach *et al*., 2005), but is not unique to this species and may also be seen occasionally in humans. Animals recovering from leptospirosis may become asymptomatic carriers harbouring virulent leptospires in the renal tubules for extended periods and shedding infectious leptospires into the environment (Levett, 2001). Susceptible animals acquire the

infection by direct or indirect contact with urine or tissues of infected animals. Other species such as mice (*Mus musculus* and other *Mus* species) and rats (mainly *Rattus norvegicus* and *R. rattus*) serve as reservoirs for their host-related serovars (mice for L. Ballum, L. Icterohaemorrhagiae and rats for L. Copenhageni) (Bharti *et al*., 2003). They usually do not show signs, but harbour leptospires in their kidneys, becoming an important source of infection for humans or other animals through direct contact with infected urine or indirectly via contaminated feed and water with infected urine (Sykes *et al*., 2011).

2.5.1 Clinical signs in dogs

The severity of clinical signs is influenced by a dog's age, vaccination status, the inherent virulence of a particular leptospiral serovar, as well as the route and degree of exposure (Adler and Moctezuma. 2010). In peracute to subacute disease, dogs may die without clinical signs. Such dogs commonly present with loss of appetite, fevers of 38.5° - 40ºC, severe myalgia and reluctance to move, stiffness, shivering, progressive weakness and depression (Grooms, 2006). Dogs may vomit and/or have diarrhoea resulting in rapid dehydration and excessive thirst. Injected mucus membranes are typical, often with widespread petechial and ecchymotic haemorrhages. Icterus is uncommon, and it occurs more frequently in dogs infected with L. Icterohaemorrhagiae (Adler and Moctezuma, 2010)

Dogs usually have conjunctivitis and congested oral mucus membranes. There also can be a dry spontaneous cough accompanied with difficulty in breathing. In addition, dogs may have frequent urination, often with haematuria and, later, anuria may occur. There also can be haematemesis, haematochezia, melena and epistaxis; eventually, infected dogs may have cold extremities and, finally, death in untreated cases (Sykes *et al*., 2011). Acutely ill dogs also may have gray-coloured stools, yellow skin and eyes, and develop chronic weight loss. In

chronic cases, there may be no apparent illness, or only fever of unknown origin and mild to severe conjunctivitis (Bharti *et al*., 2003).

2.5.2 Immunity to infection

Dogs in different parts of the world may be infected by many different serovars, but the local prevalences vary. Vaccines currently used in dogs in most countries contain the serovars L. Canicola and L. Icterohaemorrhagiae. In the newer vaccines, L. Grippotyphosa and L. Pomona have been added. The development of protective immunity to leptospirosis is believed to be associated with opsonising and bactericidal antibodies directed to the LOS and associated protein antigens (Adler and Moctezuma, 2010). Older vaccines may produce immunity which is adequate to suppress systemic invasion by homologous serovars, but not to prevent colonization of a dog's kidneys, resulting in renal carrier states (Rohrbach *et al*., 2005).

The localization of leptospires in the proximal tubules of the kidney, and survival in cerebrospinal fluid (CSF) and vitreous humour of the eye in some infected animals reflects the inability of antibodies to penetrate into those sites without causing inflammation (Sykes *et al*., 2011). It should be recognized that protection by vaccines is serovar specific and, to a lesser extent, serogroup specific. Protection against leptospirosis is related to the level of agglutinating and/or opsonising antibodies. Despite the availability of vaccines for several decades, the duration of vaccine induced immunity is not known since data from long-term challenge studies are not available.

The most commonly used serological test for leptospirosis is the *Leptospira* Microscopic Agglutination Test (L-MAT). It detects IgM responses well, but it is not as efficient in detecting IgG responses. The decline of L-MAT titres often commences about 16 weeks post-vaccination, but low titres may not indicate lack of immunity since anamnestic responses may be sufficient to engender protection against clinical illness. The protection afforded by whole cell bacterins is short (anecdotally, about 9 months) suggesting that dogs at high-risk of infection require boosters at least twice a year. As indicated above, the question whether or not to vaccinate an animal must take into account the leptospiral serovars in a particular region and ascertain that the appropriate serovars are contained in a vaccine. As with other bacterins, adverse vaccine reactions may occur which are likely due to the effects of the leptospiral LOS which is different in structure from other Gram-negative bacterial LPS. Current vaccine research is concerned with subunit products and is aimed at determining which fraction(s) of the leptospiral cell wall are immunogenic and protective without being toxic to the animal. An ideal vaccine would reduce the rate of adverse reactions, yet provide protection against both homologous and heterologous serovars.

Immunity in leptospirosis is predominantly humorally mediated in humans and most animal species, including dogs, pigs, guinea pigs and hamsters (Miller *et al*., 2011). Evidence for this arises from many findings showing that immunity can be passively transferred by convalescent human or animal serum, by experimentally produced antiserum, or by appropriate monoclonal antibodies (Mabs) such as those directed against leptospiral LPS (Jost *et al*., 1986), which have been shown to protect guinea pigs, hamsters, dogs and monkeys against lethal infection.

The protective capacity of such sera correlates with the levels of agglutinating, anti-LPS antibodies. MAbs against LPS are agglutinating and opsonic (Adler and Faine, 1983) and in the presence of specific antibodies, leptospires are readily phagocytosed by macrophages and neutrophils, both *in vitro* and *in vivo*. In addition, antibodies readily lyse leptospires in the presence of complement. LPS is highly antigenic, in both native form in whole or disintegrated leptospires, or in chemically purified form and immunization with purified LPS or LPS components elicits protective immunity (Jost *et al*., 1989; Masuzawa *et al*., 1996; Midwinter *et al*., 1990). Significantly, LPS is a major antigen recognised in human and animal convalescent sera and immunity following naturally acquired infection and is restricted to serovars with serologically related LPS. Early work on immunity amongst antigenically unrelated serovars (Kemenes, 1964; Plesko and Hlavata, 1971) did not identify the responsible antigens, but intriguingly may have involved one or more of the outer membrane proteins or lipoproteins identified many years later by molecular studies.

Vaccines for humans and animals have been used since the 1920s; almost all of them were prepared from whole leptospiral cells killed by a variety of methods, including heat, formalin, phenol, irradiation etc (Faine *et al*., 1999). The use of undefined, live avirulent, attenuated, or saprophytic leptospires has not gained acceptance. Many of these early preparations were too reactogenic for widespread human use (Adler and Moctezuma, 2010). Attempts to reduce reactogenicity have included the use of protein-free media for growth of leptospires and the use of sub-cellular fractions, the active component of which was almost certainly LPS.

Nevertheless, human vaccines containing whole killed leptospires have been used successfully in China following floods resulting in exposure of large populations to leptospirosis (Chen, 1985), and in Japan and Vietnam. Generally, vaccines contain two or

more locally prevalent serovars. For example, a trivalent killed vaccine was recently developed in Cuba following the unavailability of an imported vaccine (Martı´nez *et al*., 2004). In all cases, repeated annual revaccination is recommended to retain immunity. As with natural infection, immunity is restricted to antigenically related serovars.

Vaccination of livestock can reduce urinary shedding and risk to human handlers especially when accompanied by appropriate education programs, awareness, and hygiene in the community, and support from the authorities responsible for administration of human and veterinary public health. Commercial *Leptospira* vaccines are available globally for cattle, pigs and dogs but vaccination has proved to be only partially effective, due in part to the serovar restricted nature of vaccine induced immunity and the potential presence of local serovars other than those included in the vaccines (Sykes *et al*., 2011). A successful vaccination program requires continued epidemiological studies to assess the incidence of different *Leptospira* serovars in a given population. Most vaccines developed in the past have consisted of whole cell bacterins containing leptospires inactivated by chemical means or heat (Wang *et al*., 2007). Very early studies by Broom (1959), showed that hamsters could be protected with phenol-treated L. Canicola cultures, while Brunner and Meyer immunized dogs and hamsters with a bacterin containing L. Canicola and L. Icterohaemorrhagiae and showed only serovar-specific protection.

Canine vaccines generally, but not exclusively, contain serovars L. Canicola and L. Icterohaemorrhagiae. Vaccines generally protect against disease and renal shedding under experimental conditions, but transmission of serovar L. Icterohaemorrhagiae from immunized dogs to humans has been reported (Duncan *et al*., 2012). Moreover, immunized dogs may be infected with serovars other than those contained in commercial vaccines (Prescott, 2008).

Recent vaccines may also include serovars L. Grippotyphosa and L. Pomona in addition to the traditional vaccine strains, in response to the increasing incidence of canine infection with these serovars (Midence *et al*., 2012).

The first published report of protective immunity elicited by defined protein antigens (Haake *et al*., 1999) involved immunization of hamsters with *E. coli* membrane fractions containing a combination of OmpL1 and LipL41. This preparation induced significant protection against homologous challenge with *L. kirschneri* serovar L. Grippotyphosa. No protection was observed with either antigen administered alone. Interestingly, non membrane associated forms of the antigens were not protective, even when used in combination, leading the authors to suggest that the manner in which these proteins associate with the membrane is important in the induction of a protective immune response (Adler and Moctezuma., 2010).

Since that report, several additional OMPs have been evaluated, with varying degrees of success. The leptospiral outer membrane is dominated by the lipoprotein LipL32 which makes up more than 50% of both the outer membrane subproteome (Cullen et al., 2002) and the surfaceome (Cullen *et al*., 2004; Cullen *et al*., 2005). Despite this fact, immunization with LipL32 has yielded equivocal results. For example, immunization of gerbils with a recombinant adenovirus expressing LipL32 yielded statistically significant protection (Branger *et al*., 2001); however, the biological significance of this result must be tampered by the fact that 50% of control animals also survived. A follow up which used lipL32 DNA immunization (Branger *et al*., 2005) likewise found survival rates of 60% compared with 35% in controls, but the difference was not statistically significant (Adler and Moctezuma, 2010). Similar problems arose when assessing the results of immunizing hamsters with LipL32 expressed in *M. bovis* BCG (Seixas *et al*., 2007).

LipL32 is expressed during infection and elicits an antibody response (Flannery *et al*., 2001) but these antibodies are apparently not protective. One can envisage protection arising through two possible mechanisms. Firstly, antibodies could opsonise leptospires for phagocytosis. In the case of LipL32, antibodies may be directed against epitopes which are not accessible on the leptospiral surface (Picardeau *et al*., 2008). Neither the structure nor the membrane topology of LipL32 is known, so this possibility remains speculative. Alternatively, antibodies may act to inhibit or neutralise some biological activity. LipL32 has clearly been shown to bind mammalian extracellular matrix (ECM) proteins (Hauk *et al*., 2008; Hoke *et al*., 2008). However, *Leptospira* appears to possess a degree of redundancy in ECM adhesion, as a lipL32 mutant retained ECM-binding activity, virulence for hamsters and the ability to colonise rat kidney (Murray *et al*., 2009b). The short comings of these various vaccine studies highlight the importance of a proper virulent challenge in the evaluation of vaccines.

The Lig proteins were identified as major components of the leptospiral surface which are not expressed under normal *in vitro* growth conditions (Matsunaga *et al*.,2003; Matsunaga *et al*., 2005). As for LipL32, immunization studies with the Lig proteins have yielded variable results. (Koizumi and Watanabe, 2004) showed >90% protection for C3H/HeJ mice immunized with LigA and/or LigB, but however, mice are not recognised as animal models for leptospirosis. A study with LigA in hamsters claimed efficacy (Palaniappan *et al*., 2006), but control animals showed 75% survival.

A subsequent attempt to immunize hamsters with DNA encoding LigA also claimed protection; however, a proper statistical analysis of those data does not support the claim of enhanced survival. Despite these shortcomings, recent work has shown unequivocal

protection of hamsters with the C- terminal portion of LigA, but interestingly not with LigB (Silva *et al*., 2007). Despite the finding that immunization did not confer sterilising immunity, LigA appears to offer the most promising prospect for a recombinant protein vaccine.

2.5.3 Leptospirosis: The zoonosis

Leptospirosis in humans is always acquired from an animal source; human-to-human transmission is practical non-existent and the disease is regarded globally as a zoonosis. Pathogenic leptospires live in the proximal renal tubules of the kidneys of carriers, although other tissues and organs may also serve as a source of infection (Adler and Moctezuma, 2010). From the kidneys, leptospires are excreted in urine and may then contaminate soil, surface water, streams and rivers. Infections of animals or humans occur from direct contact with urine or indirectly from contaminated water. The carriers may be wild or domestic animals, especially rodents and small marsupials, cattle, pigs and dogs (Adler and Moctezuma, 2010). Almost every mammal (including aquatic mammals) and marsupial worldwide has been shown to be a carrier of leptospires. Humans almost never become chronic carriers, but suffer acute infections, sometimes with longer term sequel. Leptospirosis in humans can vary in severity according to the infecting serovar of *Leptospira*, and the age, health and immunological competence of the patient. It ranges from a mild, influenza-like illness to a severe infection with renal and hepatic failure, pulmonary distress, and death (the classical Weil's disease).

There is a worldwide occupational association, especially in developed countries, with agriculture and animal production (cropping, dairy farming, pig production, abattoirs) and a universal risk from rodent-carrier mediated infection, especially prevalent in tropical countries, where many serovars may be present in a locality. There are characteristic

associations of particular serovars with certain species of animals as carriers, but the association is not absolute and the molecular basis for this so-called maintenance host ‘‘specificity’’ is unknown. The renal carrier state is thus a key component which is central to the persistence and epidemiology of leptospirosis. Leptospires colonise the surfaces of renal proximal tubular epithelial cells. The molecular basis for this bacterial-cell association is unknown. Specific adhesion factors of leptospires for host components have been described, but their role in disease has not been elucidated (Picardeau *et al*., 2008). For example, the fibronectin-binding protein LigB is not necessary for virulence, while the major surface protein LipL32 binds host ECM, but is not required for either acute infection or kidney colonisation (Bulach *et al*., 2006). Excretion in urine may be intermittent or continuous and the urinary concentration of bacteria may be as high as108/ml. Leptospires do not survive well in acid urine, but remain viable in alkaline urine (Bulach *et al*., 2006). Consequently, herbivores and animals whose diet produces alkaline urine appear to be relatively more important as shedders than are producers of acid urine.

Leptospirosis commences suddenly with headache, fever (typically 39°C) malaise, myalgia, conjunctival suffusion and sometimes a transient rash. Thereafter the illness may be mild and self-limiting or severe and fatal. The mild type may be serious and incapacitating, but seldom leads to kidney or liver failure, haemorrhages or death. This description is applicable worldwide to illness characteristically seen commonly in infection with serovar L. Hardjo, but also with others. On the other hand, infection with some of these usually milder serovars can occasionally lead to severe leptospirosis which may be life threatening. For example, human infection with Pomona may lead to renal failure, while severe cholecystitis has been reported for L. Hardjo infections (Wang *et al*., 2007).

The initial symptoms may be followed by transient remission, which may then proceed to an exacerbation and include ‘‘aseptic’’ meningitis, renal failure and abdominal or chest pains, reflecting the generalised pathology. Clinical differential diagnosis is required between leptospirosis and severe influenza, viral meningitis, acute abdominal conditions or glomerulonephritis. Recovery is usually complete but weakness, tiredness, depression, and even psychosis may prevent a return to normal living for weeks or months (Sykes *et al.*, 2011). Reports of chronic or recurrent leptospirosis have usually not been adequately proven or investigated, but there is sufficient anecdotal evidence for these claims to be taken seriously (Picardeau *et al.*, 2008). The cellular and molecular basis for chronic leptospirosis remains unknown. A clear association was described between uveitis following leptospiral infection and the presence of leptospires (detected by PCR) in the anterior chamber of the eye (Chu *et al.*, 1998). Severe leptospirosis characteristically is seen in infections with serovars L. Icterohaemorrhagiae, L. Copenhageni, L. Lai and others. The source of infection is most commonly rats or other rodents. The illness worsens, usually rapidly after onset, so that renal failure may occur within 7–10 days, sometimes accompanied or followed by skin and mucosal haemorrhages, jaundice, haemoptysis, pulmonary haemorrhages, or liver failure, leading to death if untreated (Chu *et al.*, 1998). Case fatality rates approaching 20% have been reported (Bharti *et al.*, 2003; Levett, 2001).

A more recently recognised respiratory manifestation involves severe pulmonary oedema and haemorrhages, which have been the main cause of death in some epidemics (Picardeau *et al.*, 2008). As with mild leptospirosis, chronic, long-term sequel have been reported, but frequently not investigated fully. The host and microbial factors which may lead to long-term persistence are unknown. Leptospirosis during pregnancy carries the risks of intrauterine infection and foetal death (Bulach *et al.*, 2006).

2.6 Diagnosis and typing of isolates

Because of the wide diversity of clinical signs, diagnosis of leptospirosis is difficult and depends upon a variety of laboratory assays such as detection of specific antibodies by microscopic agglutination test (MAT), indirect hemagglutination assay (IHA) or by immuno-enzymatic assays (ELISA). Leptospires or their components may be detected in urine or tissues by culture, dark field microscopy, immuno-staining or PCR (Bharti *et al*., 2003; Faine *et al*., 1999; Levett, 2001). The MAT is the most widely used diagnostic test. It has the advantage of being specific for serovars, or at least serogroups, but it cannot discriminate between antibodies resulting from infection or vaccination; this may cause particular problems in animals, for example in screening for disease status for import or export.

The criterion for considering a result indicative of current *Leptospira* infection is usually accepted as a high single MAT titre of 400 in the presence of clinical signs and appropriate history of animal contact, or a four-fold rise in titre in paired serum samples (Faine *et al*., 1999). Both the sensitivity and specificity of the MAT are very high. However, the MAT may also present problems because of the requirement for live cultures of different *Leptospira* serovars prevalent in a particular geographical area. In order to achieve maximum reliability and standardisation, laboratories are encouraged to obtain a collection of serovars from a certified reference laboratory and to participate in quality assurance programs such as the International Leptospirosis MAT proficiency test scheme organized by the International Leptospirosis Society (ILS). Maintenance of live cultures of leptospires may also pose a health risk to laboratory personnel, therefore hygienic measures should always be followed.

ELISA tests have been developed using a wide variety of antigen preparations, from leptospiral sonicates to recombinant lipoproteins such as LipL32, LigA, or the outer

membrane porin OmpL1 (Sykes *et al*., 2011). The assay obviates the need for maintenance of live cultures and is amenable to automation. However, sensitivity and specificity do not match those of the MAT, and reliance on ELISA alone is not recommended. Other antibody detection methods include macroagglutination, latex agglutination, lateral flow assays and IgM dipstick (Adler and Moctezuma, 2010).

Detection of leptospires by culture constitutes the definitive diagnosis; however, it is hampered by slow growth rates of some *Leptospira* strains and the long incubation periods before an isolate is established in culture. Successful isolation of *Leptospira* requires fresh tissue, blood or urine samples, prior to initiation of antibiotic treatment, usually inoculation of at least two tenfold dilutions of tissue fluid or homogenate, and depending on the contamination level, 5-fluoruracil or other selective antimicrobial agents to inhibit contaminants (Faine *et al*., 1999; Levett, 2001).

Incubation for up to 13 weeks at 30° C with weekly examination by dark field microscopy (DFM) is necessary before cultures can be discarded as negative. For this reason, culture is not considered useful as a routine test for diagnosis of individual patients, but remains important for epidemiological purposes. Other alternatives, such as DFM, immunofluorescence, antigen ELISA or immunoprecipitation, for detection of leptospires in urine, blood or other tissues, lack sensitivity and specificity.

Several PCR protocols for detection of leptospiral DNA in clinical material have been developed since the 1990s; most of them reported high sensitivity. However, only two have been extensively evaluated in clinical studies (Brown *et al*., 1995; Merien *et al*., 1995) and gained widespread use for diagnosis. The protocol of Merien *et al*. (1995) is a genus-specific assay which amplifies DNA from both pathogenic and non-pathogenic serovars. On the other

hand, the approach described by Gravekamp *et al*. (1993) and evaluated by Brown *et al*. (1995), requires two sets of primers in order to detect all species containing pathogens. Improved sensitivity has been achieved by quantitative- PCR either using TaqMan probes (Slack *et al*., 2007) or SYBR green fluorescence (Levett *et al*., 2005).

The characterization of leptospiral isolates has evolved from the time consuming cross agglutination absorption protocols (Faine *et al*., 1999). More reliable and robust modern methods include restriction fragment length polymorphism (RFLP) analysis (Herrmann *et al*., 1992), 16S rRNA sequencing (Morey *et al*., 2006), detection of variable number of tandem repeats (VNTR) (Majed *et al*., 2005), and more recently by multiple locus sequence typing (MLST) (Ahmed *et al*., 2006; Thaipadungpanit *et al*., 2007). MLST promises a more straightforward characterization method for *Leptospira* isolates which is amenable to standardisation through online databases (http://leptospira.mlst.net/), allowing ready access to current molecular epidemiology for almost every laboratory worldwide.

2.7 Treatment and control in dogs.

The aims of treatment for acute cases of canine leptospirosis are to control the infection before irreparable damage is done to the liver and kidneys, and to suppress the leptospiruria (Sykes *et al*., 2011). Severely ill, acute cases require a high degree of supportive care for survival; the prompt administration of fluids is essential. The prognosis is guarded for patients with acute renal failure and/or liver disease. Owners should be advised that leptospirosis is a zoonotic disease that is spread mainly by the urine of infected dogs (Midence *et al*., 2012). An infected dog's housing and outside areas need to be treated with a

suitable disinfectant. Also, dogs should avoid muddy, stagnant water and rodents. Rodent control should be instituted. Vaccination is recommended in endemic areas.

Dogs usually recover after 2 weeks, if treated promptly with antibiotics and intravenous fluids. However, if kidney or liver damage is severe the infection may be fatal. Successful treatment depends on an assessment of the severity of the dog's disease. Initial antimicrobial therapy, where there is evidence of renal dysfunction and/or leptospiremia, should include the use of procaine penicillin G (40,000 to 80,000 Units per kg IM, sid, or in divided doses, bid) until kidney function returns (Sykes *et al*., 2011). Alternative drugs such as ampicillin or amoxicillin also may be used in place of penicillin. Elimination of leptospires from the renal interstitial tissues to control the carrier state is best achieved with dihydrostreptomycin (10 to15 mg per kg, IM, bid for 2 weeks) or streptomycin. Doxycycline is not formally approved, but oral administration of 5.0 mg per kg SID has been proposed. Aminoglycosides cannot be used in patients until kidney function has been restored.

Prevention of leptospirosis without vaccination is quite difficult (Midence *et al*., 2012). Measures for occupational hygiene such as protective clothing and avoidance of splash from urine or water are often useful but hard to implement because they impede work or are unacceptable to both workers and employers (Adler and Moctezuma, 2010). For example, it is not practicable to advise dwellers in tropical villages to avoid hazardous activities like contact with pigs and other livestock or dogs, and walking or working in wet conditions, including in soil or water, such as rice paddies, contaminated by the animals' urine (Sykes *et al*., 2011).

Control methods should, therefore, include vaccination; special attention to kennel sanitation to eliminate contact with potential sources of infected urine; knowledge that high risk dogs

are hunter breeds, show dogs, and other dogs with access to water such as ponds; institute rodent control of households and kennels (Midence *et al*., 2012).

CHAPTER III

3. MATERIALS AND METHODS

3.1 Study location and sampling of specimens

The study was carried out in one urban area (Harare) and five rural communities (Marumani, Machuchuta, Ndhlovu, Kariba and Malipati) in Zimbabwe. In Harare, serum samples were obtained from blood collected from dogs presented to veterinary private practices for routine elective surgery or ill-health. Routine vaccination against leptospirosis is done in most of the urban areas in the country and for the purposes of this study, only dogs with no given history of vaccination were included in the study.

The Animal and Wildlife Area Research and Rehabilitation TRUST (AWARE TRUST) has been conducting dog spay and castration campaigns and, sample collection in randomly selected rural communities of Zimbabwe, namely Marumani, Machuchuta, Ndhlovu, Kariba and Malipati. Owing to an access to large dog serum samples, the rural communities where AWARE TRUST was conducting these activities were selected for the study. Hence, serum samples collected by AWARE TRUST just prior to ovariohysterectomy and orchidectomy of apparently healthy rural-owned and stray dogs with unknown medical histories were used for the study. Currently rural dogs are not routinely vaccinated against leptospirosis.

3.2 Testing for canine leptospirosis

The Canine *Leptospira* Antibody Test kit (ImmunoComb®, Biogal-Galed Laboratories, Israel) designed to determine dog serum IgG antibody titres to four different serovars (L. Canicola, L. Grippotyphosa, L. Icterohaemorrhagiae and L. Pomona) of *Leptospira interrogans* was used for this study. The ImmunoComb® test is an enzyme labelled 'dot

assay' modified ELISA (Biogal-Galed Labs in Israel). Testing of the serum samples was done according to the manufacturers' instruction manual (www.biogal.co.il). Briefly, a mixture of the *L. interrogans* antigens was used to screen for the presence of antibodies to the four *Leptospira* serovars. Before conducting the test, the developing plates were incubated for 22 minutes at 37° C. The test was then performed at room temperature (20°-25°C) in a 72-well developing plate (12 columns x 6 rows) where 5µl of the serum deposited only into each well of the first row, was mixed with the antigens adsorbed onto the lower portion of the Comb and, allowed to stand for five minutes. The Comb was transferred to corresponding wells of the second row where non-bound antibodies were washed off. The Comb was then inserted into the wells of the third row which contained an enzyme labelled anti-dog IgG antibody that would bind to the antigen-antibody complex at the test spots and was allowed to stand for five minutes. After two more washes in wells of the next two rows, the Comb was transferred for five minutes to the corresponding wells of the last row where a colour result developed via an enzymatic reaction. Upon completion of the colour development in cells of the last row, the Comb was moved back to cells of the fifth row for two minutes to facilitate colour fixation. The Comb was removed and let to dry for 10 minutes.

The threshold for determining seropositivity was based upon the manufacturers' recommendations. The bottom spot on the Comb gave the result of the anti *Leptospira* serovars mix IgG antibodies reaction in the samples tested. The colour intensity of the bottom anti *Leptospira* serovars mix spot was then compared with that of the upper Positive Reference spot. A clear, visible purple-grey dot indicated a positive response. The results were read with a calibrated graded (S0 to S6) colour CombScale provided with the kit. A scale of S3, which is equivalent to a positive immune response at a titre of 1:400 by the Microscopic Agglutination Assay (MAT), was considered as the 'cut-off' level of IgG

antibodies (www.biogal.co.il). Hence, in this study serum samples giving a CombScale score of ≥S3 (≥ 1:400 titre) were considered as positive to the tested *Leptospira* serovars.

3.3 Laboratory evaluation for clinicopathological features suggestive of leptospirosis: hepatic damage and renal insufficiency

Based on laboratory findings of hepatic damage and renal insufficiency as suggestive of leptospirosis (Adler and Moctezuma, 2010), collected dog serum samples were evaluated for elevated liver enzymes and/or urea and creatinine. Seropositive and some randomly selected seronegative samples were further investigated for hepatic damage and renal insufficiency. Hepatic damage was determined by measuring liver enzyme activity; that is Aspartate aminotransferase (AST; reference value < 30 IU/L), Alkaline phosphatase (ALP; reference value < 100 IU/L) and Alanine aminotransferase (ALT; reference value < 30 IU/L) while renal insufficiency was determined through measuring blood urea nitrogen (BUN; reference value < 6 mmol/L) and serum creatinine (reference value < 150 mmmol/L). An automated chemistry analyser (Humastar 180®- Human GmbH, Wiesbaden, Germany) from a private laboratory was used to measure liver enzyme activity, creatinine and blood urea nitrogen. Reference values for these parameters were adopted from Diagnopath laboratory, Private limited.

3.4 Questionnaire surveys on dog zoonoses

A cross-sectional questionnaire-based study was employed to investigate public knowledge on dog zoonoses. A systematic random sampling technique was used to select dog owners attending private veterinary practice in Harare. Self-completion questionnaires were distributed to at least 10% of dog owners' attending each veterinary practice. The

questionnaire was designed to obtain information on dog owners' knowledge and awareness of dog zoonoses with particular emphasis on canine leptospirosis and, their behavioural practices that may lead to increased risk of canine leptospirosis transmission. A standard questionnaire with multiple choice and open-ended questions was used.

3.5 Data analysis

The recording and editing of the data was performed using Microsoft Excel®. Statistical analyses were performed using Stata SE/9.0 for Windows (Stata Corp. College Station, TX, USA, 2009). The total number of sero-positive animals was calculated from the total number of samples tested over the study period and expressed as a percentage. Sero-positive animals were examined in relation to sex and location. Sex and location categories were generated as follows: two for sex (male and female) and two for location (urban and rural). The Chi-square test was used to measure differences in proportions between generated categories and values of $P < 0.05$ were considered as significant. Seropositivity was also analysed according to hepatic damage and renal insufficiency. Association between seropositivity and hepatic damage and/or renal insufficiency was evaluated by calculating the Chi-square test, relative risk (RR) and the 95% confidence interval (CI) using win episcope version 2.0. Data analysis on questionnaire responses was focused on generation of descriptive statistics (frequencies/proportions) related to dog owners' knowledge and awareness of dog zoonoses with particular emphasis on canine leptospirosis and, their behavioural practices that may lead to increased risk of canine leptospirosis transmission.

CHAPTER IV

4. RESULTS

4.1 Leptospirosis seroprevalence

The distribution of sampled dogs and their seroprevalence according to different categories are shown in Table 1. A total of 250 dog serum samples were collected and the overall seroprevalence was 15.6%. Overall, urban dogs from Harare recorded a significantly (X^2 = 7.9, $P < 0.05$) higher seroprevalence compared to rural dogs. Urban female dogs recorded a significantly (X^2 = 9.5, $P < 0.05$) higher seroprevalence compared to rural female dogs but there was no significant difference between urban male (X^2 = 0.61, $P > 0.05$) and rural male dogs. Overall, female dogs (16.4%; 10.2-22.7) recorded a relatively higher seroprevalence compared to males (14.7%; 8.2-21.1), but the difference was not statistically different (X^2 = 0.149, $P > 0.05$). However, when categorized according to location, rural male dogs recorded a relatively higher seroprevalence compared to rural females (X^2 = 0.39; $P > 0.05$) while urban female dogs had a relatively higher prevalence compared to urban males (X^2 = 1.7; $P > 0.05$) but the differences were not statistically significant. For the rural dogs the seroprevalence varied from 5.9% - 17.6% but the differences were not significant.

The majority (69.2%) of dogs which tested positive had a titre of 1:400 with 45% of positive urban dogs recording a titre of 1:800 and above (Table 2). One each of the positive urban dogs had a titre of 1:1600 and 1:3200 while none of the positive rural dogs had a titre above 1:800 (Table 2).

A total of 80 dogs were evaluated for hepatic damage and renal insufficiency and 22.5% had both hepatic damage and renal insufficiency while 10% had hepatic damage only and less

than 3% had renal insufficiency only (Table 3). These dogs had median values (range) of 25 for BUN (3-77 mmol/L), 202 for creatinine (47-1309 ummol/L), 72 for AST (36-271 IU/L), 284 for ALP (87-5924 IU/L) and 76 for ALT (38-372 IU/L). Seropositive dogs with hepatic damage and/or renal insufficiency were all urban dogs while none of the rural seropositive dogs had hepatic damage and/or renal insufficiency. Of these 20 urban seropositivity dogs, the majority (33.33%) had both hepatic damage and renal insufficiency followed by those with hepatic damage only and lastly renal insufficiency only (Table 3). The seropositive urban dog that recorded the highest titre of 1:3200 also recorded the highest ALP value of 5924 IU/L. Overall, the results demonstrated a significant association ($X^2 = 8.9$; $P < 0.01$) between seropositivity and hepatic damage and/or renal insufficiency and those with hepatic damage and/or renal insufficiency were approximately 2 times ($RR = 1.96$; $1.3 < RR < 3.0$) more likely to be seropositive for leptospirosis.

4.2 Questionnaires responses

A total of 151 (75.5%) dog owners of 200 contacted responded. Fifty-two percent (79/151) were males and 48% (72/151) were females with most respondents (80.8%; 122/151) being aged 21 years and above. Most respondents (87.4%, 132/151) were from non-medical professionals, followed by those from the veterinary (8.6%; 13/151) and human medical (4%; 6/151) profession. Most respondents (89.4%; 135/151) had kept dogs for over a year with the majority keeping dogs for protection (82.8%; 125/151), followed by pet (53%, 80/151) and breeding (4%, 6/151). The number of dogs owned ranged from 1-16 with most (78.1%; 118/151) owning 1-3 dogs and the median number of dogs per respondent was 2. Most respondents (60.3%; 91/151) had been visiting a veterinarian for over a year. Reasons cited

for visiting a veterinarian were; treatment of sick dog (83.4%; 126/151), vaccinations and deworming (80.8%; 122/151), and education and guidance on dog health (45.7%; 69/151).

When asked generally on their awareness of dog zoonoses, 78.8% (119/151) of dog owners were aware (Table 4). Of the 78.8% who were aware of zoonoses in dogs a higher proportion knew rabies as a zoonotic disease in dogs compared to other named zoonoses (Table 4). Other cited zoonoses were worms (6.7%), leptospirosis (5%), brucellosis (1.7%), ringworm (0.8%) and toxoplasmosis (0.8%). Those who named leptospirosis as a zoonotic disease in dogs were all from the veterinary profession. A few dog owners responded that anthrax (0.8%), fleas (0.8% and ticks (0.8%) were zoonoses transmitted from dogs.

Table 1: The distribution of Leptospire seroprevalence according to sex and location

Category	**Level**	**Number tested**	**Number Positive**	**% seroprevalence and 95% confidence interval**
	All animals	250	39	15.6 (11.0 – 20.2)
Urban	Females	42	13	31.0 (17.1 – 44.9)
	Males	38	7	18.4 (6.1 – 30.7)
	Overall	80	20	25.0 (15.6 – 34.4)
Rural	Females	92	9	9.8 (3.7 – 15.9)
	Males	78	10	12.8 (5.4 – 20.2)
	Overall	170	19	11.2 (6.5 – 15.9)
Rural	Marumani	34	2	5.9 (-1.9 – 13.7)
	Machuchuta	24	2	8.3 (-2.7 – 19.3)
	Ndhlovu	54	5	9.3 (1.5 – 17.1)
	Kariba	24	4	16.7 (1.0 – 32.4)
	Malipati	34	6	17.6 (3.9 – 31.3)

Table 2: The distribution of Leptospire seroprevalence according to location and titre

Category	**No. of positive samples**	**Titre**			
		1:400 (%)	1:800 (%)	1:1600 (%)	1: 3200(%)
Rural	19	16 (84.2)	3 (15.8)	0	0
Urban	20	11 (55.0)	7(35.0)	1(5.0)	1(5.0)
Overall	**39**	**27 (69.2)**	**10 (25.6)**	**1 (2.6)**	**1 (2.6)**

Table 3: The number and percent of dogs evaluated for hepatic damage and renal insufficiency according to seropositivity

Category	**No. leptospire seropositive (%)**	**No. leptospire seronegative**	**Total (%)**
Hepatic damage and renal insufficiency	13 (33.33)	5	18 (22.5)
Hepatic damage only	5 (12.82)	3	8 (10.0)
Renal insufficiency only	2 (5.13)	0	2 (2.5)
No hepatic damage/renal insufficiency	19 (48.72)	33	52 (65.0)
Total	**39**	**41**	**80**

Table 4: Dog owners' awareness responses of dog zoonoses

Variable	**Number**	**Percent**
General dog zoonoses (n = 151)	119	78.8
Named zoonoses (n = 119)		
Rabies	110	92.4
Worms	8	6.7
Leptospirosis	6	5.0
Brucellosis	2	1.7
Ringworm	1	0.8
Tetanus	1	0.8
Toxoplasmosis	1	0.8

CHAPTER V

5. DISCUSSION

The reference method for serological diagnosis of leptospirosis is the microscopic agglutination test (MAT) (Levett, 2001). However, because of the complexity of the MAT, rapid screening tests for leptospiral antibodies in acute infection have been developed. One such test, the indirect enzyme-linked immunosorbent assay detecting the immunoglobulin G (IgG), the ImmunoComb® ELISA kit (Biogal-Galed Laboratories, Israel) used previously in other studies (Odentsetseg *et al*., 2005) was used in the present study. Previous studies (Cumberland *et al*., 1999; Jimenez-Coello *et al*., 2008) reported a good concordance between the ELISA IgG and the MAT in the diagnosis of leptospirosis and suggested that it could potentially substitute the MAT because it was rapid, sensitive and technically less demanding. Because the MAT relies on the use of live attenuated antigens which require frequent sub-culturing, it potentially poses a risk of infection to the laboratory personnel (Odentsetseg *et al*., 2005).

Both rural and urban dogs sampled during the present study had no given history of vaccination against leptospirosis. Currently, only urban dogs in the country are routinely vaccinated using the bivalent vaccine containing the serovars L. Canicola and L. Icterohaemorrhagiae. Generally, vaccinated dogs have negative titres or titres against these serovars not exceeding 1:320 that persist for a few months after vaccination (Hartman *et al*., 1984). Hence, due to uncertainty on the vaccination status of some of the urban dogs, a more liberal cut-off point of titres ≥ 1:400 for *Leptospira* seropositive dogs was adopted (Levett, 2001). The ImmunoComb canine *Leptospira* ELISA kit used has a high sensitivity (80%), thus reducing the possibility of false negative reactors (www.biogal.co.il). Although cross-

reactivity between antibodies of *Leptospira* and *Borrelia* spp. has been suspected, it has never been confirmed (Shin *et al*., 1993) and furthermore, Lyme disease has not yet been detected in Zimbabwe. The most commonly incriminated serovars in canine leptospirosis have been L. Canicola, L. Icterohaemorrhagiae, L. Pomona, L. Bratislava and L. Grippotyphosa (Greene *et al*., 1998). Despite its inability to distinguish the specific serovars, the ImmunoComb ELISA kit has a mixture of antigens of the four most widespread and most common serovars in dogs; L. Canicola, L. Icterohaemorrhagiae, L. Grippotyphosa and L. Pomona. Hence, the positive results obtained in the present study are likely to indicate exposure to one or more of these common serovars. Considering that some of the seropositive dogs had very high antibody titres, this could be indicative of active infection. However, lack of serovar-specific data in the present study makes it difficult to conclude on the predominant serovar (s) and this requires further investigations.

The finding of titres of 1:3200 or more, which indicate an acute form of leptospirosis (Rentko *et al*., 1992), suggests that the infection was active in one of the dogs at the time of sampling. The highest titre detected in this study was 1:3200 (CombScale value 6) from an urban dog that also recorded the highest elevated ALP value of 5924 IU/L. This was likely to be due to an active infection and indicates that annual boosters are needed to keep the infection low.

In Zimbabwe there is limited information on canine leptospirosis. The first report was that of one confirmed case among dogs in Bulawayo urban, south-west of Zimbabwe during the period 1956-1957 (Graf, 1965). A survey in the same urban area, demonstrated the presence of the disease in two dogs diagnosed serologically and clinically and a seroprevalence of 13.7% (19/146) against the serovar L. Canicola (Banks and Pigott, 1979). The disease was also detected serologically in 2.7% of dogs but details on number and origin of the samples

and, the infecting serovar (s) were not given (Feresu, 1982). Serological results of the present study using a pooled antigen of serovars L. Canicola, L. Grippotyphosa, L. Icterohaemorrhagiae and L. Pomona points to the presence of leptospirosis in both urban and rural dogs.

The epidemiology of leptospirosis is characterized by a primary host species that acts as the reservoir for each serovar. *Leptospira interrogans* serovars L. Canicola and L. Icterohaemorrhagiae have been reported as the most prevalent serovars causing canine leptospirosis worldwide (Greene and Shotts, 1990). Dogs and rats are the reservoir species for serovars L. Canicola and L. Icterohaemorrhagiae, respectively (Greene *et al*., 1998). The widespread use of bivalent vaccines that are serovar-specific for only L. Canicola and L. Icterohaemorrhagiae has resulted in a decreased prevalence of canine leptospirosis associated with those serovars (Prescott *et al*., 2002; Higgins, 2004). However, there have been reports of the apparent re-emergence of canine leptospirosis associated with a change in the infecting serovars (Bolin, 1996). The serovars commonly reported are principally L. Grippotyphosa and L. Pomona (Ward *et al*., 2002; Ghneim *et al*., 2007) with a wide range of wild and domestic animal reservoir species that include voles, racoons, skunks, opossums, mice, pigs and cattle (Greene *et al*., 1998). In Zimbabwe, the serovars L. Canicola, L. Icterohaemorrhagiae, L. Grippotyphosa and L. Pomona have been detected serologically in pigs (Mavhenyengwa *et al*., 1999), cattle (Graf, 1965; Swanepoel *et al*., 1975; Feresu, 1987, 1988) and rodents (Dalu and Feresu, 1997). It is therefore, likely that these reservoirs together with other dogs (L. Canicola) act as sources of infection for dogs. However, rats in particular are major sources of infection for humans and dogs and it has been suggested that the serovars present in rodents in a given environment are similar to those present in dogs living in that same environment. Given an earlier high rodent seroprevalence (62.5%) and

high serovar isolation, with most being obtained from *Rattus rattus* in one of the study areas (Harare) (Dalu and Feresu, 1997), rats could be a major source of infection but further investigations are required.

As observed earlier (Adin and Cowgill, 2000; Barr, 2002; Alton *et al*., 2009), the most important risk factor for leptospirosis in the current study population was location, with urban dogs being at a higher risk for being leptospirosis seropositive than rural dogs. This may be due to a higher sampling proportion error of dogs with hepatic damage and/or renal insufficiency presented to private veterinary practitioners in the studied urban area. A higher population density of infected raccoons in urban areas of Canada has been implicated as a source of the disease for domestic dogs (Alton *et al*., 2009). Earlier studies in Zimbabwe showed a high rodent leptospirosis seroprevalence (62.5%) in Harare and rodents were suggested as important reservoirs and transmitters of the disease to dogs and humans (Dalu and Feresu, 1997). The greater odds of leptospirosis may probably reflect the higher density of dogs, people, rodents and veterinary clinics in the studied urban area. However, no rural comparative studies on rodent leptospirosis have been carried out. There is, therefore, a need for further studies to better understand the epidemiology of leptospirosis in both urban and rural settings. The failure to detect any overall association between the sex of dogs and leptospirosis seropositivity in the current study agrees with published reports of leptospirosis in other regions (Ward, 2002; Ward *et al*., 2002; Suepaul *et al*., 2010).

Infection with the serovar L. Icterohaemorrhagiae has classically been associated with either acute haemorrhagic disease or liver failure and uremia (Wohl, 1996). In contrast, patients with classic infections with serovar L. Canicola are likely to exhibit acute renal failure with

less hepatic involvement (Wohl, 1996). Infections with serovars L. Pomona, L. Grippotyphosa and L. Bratislava have recently been reported to be predominantly associated with renal involvement with less consistent hepatic involvement (Brown *et al*., 1996; Birnbaum *et al*., 1998; Adin and Cowgill, 2000; Okewole and Ayoola, 2009). The observed significant association between *Leptospira* seropositivity and hepatic damage and/or renal insufficiency may further be indicative of infection with the tested serovars. Most of the urban seropositivity dogs had both hepatic damage and renal insufficiency which could probably be attributed to either L. Icterohaemorrhagiae and/or L. Canicola infection. For those that had hepatic damage only, L. Icterohaemorrhagiae could probably be the causative agent while L. Canicola, L. Grippotyphosa or L. Pomona could not be ruled out for those with renal insufficiency only. However, since the serological test used lacked serovar-specificity and no isolation was performed, other causes of hepatic damage and renal insufficiency could not be ruled out. Currently, routine leptospirosis diagnosis is not done in the country and it is likely that the disease is missed. In view of the emerging new serovars and zoonotic risks posed by the disease, further epidemiological studies are required to identify the prevailing *L. interrogans* serovars in dogs in Zimbabwe. This will enable formulating appropriate control measures against the disease such as the use of a polyvalent commercial vaccine that includes the new serovars.

Except for respondents in the veterinary profession, results of the present study concur with earlier observations in the country (Mosalagae *et al*., 2011; Pfukenyi *et al*., 2010) which showed that most of the respondents were aware of rabies as a zoonotic disease compared to other diseases such as leptospirosis. Hence, awareness, teaching and training programmes for pet owners need to be instituted to improve disease control and reduce the public health risk of pet zoonoses such as leptospirosis and others.

CHAPTER VI

6. CONCLUSION AND RECOMMENDATIONS

Although further surveys in other areas of the country still need to be undertaken, the results from this study have shown that leptospirosis was present and represented a risk to dogs from the selected rural and urban areas in Zimbabwe. The significantly higher seroprevalence of leptospirosis in Harare compared to rural areas may be indicative of differences in area-level risk factors for the disease. Leptospirosis seroprevalence was not significantly different between male and female dogs, indicating that sex was not an important risk factor in canine leptospirosis. Leptospirosis should be included as a differential diagnosis in dogs with either hepatic damage and/or renal insufficiency as the disease affects the liver and kidneys. It is suggested that the use of *Leptospira* vaccines containing the serovars Canicola and Icterohaemorrhagiae should be continued, while investigations of other infecting serovars are undertaken, as this data is lacking. Sero-epidemiological information of infecting serovars will help in deciding canine *Leptospira* vaccine to use as vaccine induced protection is serovar specific. It is also recommended that teaching and training programmes for pet owners need to be instituted to improve disease control and reduce the public health risk of pet zoonoses.

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APPENDIX I-QUESTIONNAIRE

UNIVERSITY OF ZIMBABWE, FACULTY OF VETERINARY SCIENCECLINICAL VETERINARY DEPARTMENT

Questionnaire survey on dog ownership and knowledge of diseases transmissible by these animals to humans in Harare, Zimbabwe

A. PERSONAL INFORMATION

Q.1 Please state your gender

Male ☐
Female ☐

Q.2 Please state your age range

Less than 10 years ☐
10-20 years ☐
21-30 years ☐
More than 30 years ☐

Q.3 What is your first language (PLEASE CHECK ONE BOX ONLY)

Shona ☐
Ndebele ☐
English ☐
French ☐
Portuguese ☐
Others (Please specify) ________

Q.4 Please state your occupation ……………………………………………………

B. PET OWNERSHIP

Q.5 Which of the following pets do you own?(PLEASE CHECK ALL THAT APPLY)

Cats ☐

Dogs ☐

If your answer is cat only, proceed Q11

Q.6 How many dogs do you currently own?

Dogs ☐

Q.7 What are the age ranges of the dog (s) you currently own? (PLEASE CHECK ALL THAT APPLY)

Dogs < 1 month ☐

1-2 months ☐

3-12 months ☐

> 12 months ☐

Q.8 Where do you keep your dog (s)?

Dogs Indoor ☐

Outdoor ☐

Both ☐

Q.9 For what purpose(s) do you keep your pet (s)? (PLEASE CHECK ALL THAT APPLY)

Breeding ☐
Protection ☐
Pet ☐
Others (Please specify) ________

Q.10 For how long have you been keeping pets?

< 1year ☐
1-5 years ☐
6-10 years ☐
> 10 years ☐

C. PET HEALTH

Q.11 When did you start visiting your veterinarian?

Within the last 6 months ☐
Within the last 7 to 12 months ☐
More than a year ☐

Q.12 How often do you visit your veterinarian?

- At least once every month ☐
- At least once in every 2 months ☐
- At least once in every 6 months ☐
- At least once every year ☐
- Only when there is a problem ☐

Q.13 When would you or for what purposes do you visit your veterinarian? (PLEASE CHECK ALL THAT APPLY)

- For treatment of sick pet ☐
- For vaccinations of pet ☐
- For education and guidance on pet health ☐
- For any information related to your pet ☐

D. AWARENESS OF DISEASES TRANSMISSIBLE FROM DOGS TO HUMANS

Q.**14 Are you and/or your family members aware of any disease(s)transmitted from dogs to humans**

- Yes ☐
- No ☐

If four answer is no go to Q 17

Q.15 Name any one or more diseases, which you know, are transmitted from dog(s) to humans

…………………………

Q.16 What was your source of information? (PLEASE CHECK ALL THAT APPLY)

Veterinarian	☐
Medical (human) doctor	☐
Media	☐
Friends or relatives	☐
Others (please specify)	

Q.17 Do you have any problems with kidney or liver diseases with your dog?

Yes

No

Q.18 Did your veterinarian ever discussed with you about the diseases that cause kidney or liver problems in dogs?

Yes

No

Q.19 Are you aware how canine leptospirosis is transmitted to humans from dogs ?

Yes ☐
No ☐

Q.20 If your answer to Q19 is Yes, may you please explain how its transmitted?

……………………………………………………………………………………………….

Q.21 Does your veterinarian ever discuss with you about how this diseases is transmitted from dogs to humans?

Yes ☐
No ☐

If no go to Q24

Q.22 How often does he/she discuss with you? (PLEASE CHECK ALL THAT APPLY)

Always ☐
Only when asked ☐
Only when your were a new client ☐
Only when such diseases are diagnosed in your pet (s) ☐
Only when such diseases are diagnosed from other pets attending the same veterinary practice ☐
Only when such diseases have been reported in the media ☐
Never ☐

Q23 If your veterinarian asks you to test your pet for canine leptospirois, will you allow him?

Yes

No

Q.24 If a disease transmitted from pets to humans is diagnosed in your pet (s) and you are made aware of it, what would you do?

Give-up keeping of pets

Continue keeping pets and seek advice on such diseases

Q.25 Do you feel that veterinarians should always discuss the topic of diseases transmitted from pets to humans with you?

Yes

No

Q.26 Who do you feel should play a role in educating the public on diseases transmitted from animals to humans? (PLEASE CHECK ALL THAT APPLY)

Veterinarians

Medical (human) doctors

Media

Schools

Others (please specify)

THANK YOU for your time in completing the questionnaire. Please may you return the questionnaire to your veterinarian.

If you have any comments to make, please write them in the space below