EVALUATION OF THREE METHODS FOR ROUTINE DIAGNOSIS OF LEPTOSPIROSIS IN NIGERIA

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ABSTRACT

In Nigeria, the occurrence of leptospirosis, a zoonotic disease is not well known and is often suspected based on clinical signs, many of which are common to some febrile conditions such as babesiosis, malaria, typhoid fever and influenza. Due to poor availability of relevant laboratory facilities, the confirmation is always based on post mortem histology. Early and rapid diagnosis as well as update of information on occurrence of the disease, are essential for prevention, surveillance, good prognosis and control. Many techniques that combine sensitivity, rapidity and cost-revenue ratio assessment are not routinely used in Nigeria. However, many techniques are available in developed countries. Assessment of the morbidity and case fatality rates of leptospirosis in dogs and evaluation of three methods for use in Nigeria were carried out.

A review of 5,250 cases of different ailments in dogs presenting in two referral veterinary hospitals in Ibadan between 2005 and 2010 was carried out to assess the occurrence of leptospirosis. In addition, an evaluation of the relative sensitivity, specificity, accuracy (according to standard formulae), rapidity and cost per unit test of Dark Field Microscopy (DFM) using hyper-spectral imaging, Fluorescent Antibody Staining (FAS) and conventional Polymerase Chain Reaction (PCR) was carried out using 90 urine samples from cattle and 16, 30, 5, 2, 7, 2, 10 and 5 kidney samples from cattle, dogs, bob cats, beavers, raccoons, coyotes, foxes and opossums respectively. The rapidity was calculated as the unit time taken for each technique. The operating cost per annum and the cost per unit test for each technique were calculated using standard methods. Data of the reviewed cases and agreement of the techniques were analysed using descriptive and Kappa statistics respectively.

Leptospirosis morbidity and case fatality rates in dogs were 47.0%, and 37% respectively. The relative sensitivity, specificity and accuracy of DFM compared with FAS were 88.0%, 96.0%, 94.7%, respectively and compared with PCR were 64.7%, 73.5%, 72.5% respectively. The relative sensitivity, specificity and accuracy of PCR compared with FAS were 34.3%, 99%, 83.2% respectively. The Kappa statistics showed perfect agreement (k=0.99) between DFM and FAS, DFM and PCR and between PCR and FAS. Rapidity of the tests were 26.1 minutes, 120.0 minutes and 305.0 minutes per test for DFM, FAS and PCR respectively. The cost per unit test for DFM, FAS and PCR were ₦744, ₦1,975 and ₦7,014 respectively.

Leptospirosis morbidity and case fatality rates in dogs in this study were high and this poses a great health challenge. The Dark Field Microscopy using hyperspectral imaging technique which was the fastest and cheapest per unit test may be of benefit for routine use in Nigeria to improve diagnosis and subsequently reduce mortality.

Key words: Leptospirosis, Morbidity rate, Diagnostic technique, Accuracy, Cost

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CERTIFICATION BY SUPERVISOR

I certify that this work was carried out by Dr. Jolade Aderonke A. Sansi at the Department of Veterinary Public Health and Preventive Medicine, University of Ibadan, Ibadan.

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DEDICATION

This work is dedicated to the Almighty God (my Source) who begins a good work and is always faithful to complete it.

LIST OF ABBREVIATIONS

AVNIR	A Very Near Infra Red
CFT	Complement Fixation Test
CSF	Cerebrospinal Fluid
DFM	Dark Field Microscopy
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked Immunosorbent Assay
EMJH	Ellinghausen and McCoullough medium modified by Johnson and Harris
FAS	Fluorescent Antibody Staining
IHA	Indirect Haemaglutination
LPS	Lipopolysaccharide
MAT	Microscopic Agglutination Test
ml	millilitre
NVSL	National Veterinary Services Laboratories
OIE	Office International des Epizooties
OS	Outer Sheath
PC	Protoplasmic Cylinder
PCR	Polymerase Chain Reaction
µl	Microlitre
WHO	World Health Organization
TAE	Tris-acetate
EDTA	Ethylene Diamine Tetra Acetic Acid

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Project Definition

Leptospirosis is a febrile zoonotic and re-emerging infectious disease with protean signs that are common to a lot of other infectious diseases. It is ubiquitous and has a global geographical distribution affecting most mammals, including humans, whales and bats. The causal agent, pathogenic *Leptospira* has been detected in some reptiles, birds, and amphibians. Leptospirosis has several differential diagnoses because there are no clinical signs pathognomonic to it. Accurate and prompt diagnosis of the disease is very important for prevention, good prognosis, effective treatment, surveillance and control. Prompt detection of leptospirosis is essential as antibiotic therapy provides the greatest benefits when administered early in the infection stage (Adler *et al*. 1981, Arimitsu *et al*. 1994). Misdiagnosis is a major problem in regions where other causes of undifferentiated febrile illness and haemorrhagic fever are endemic (Alston, 1935; Adler *et al*, 1982; Arimitsu *et al*, 1982; Arimitsu *et al*, 1989). A major problem in the diagnosis of leptospirosis is the lack of an efficient, fast and economic diagnostic tool (Agunloye, 2002).

1.2 Background information and Problem Statement

Leptospirosis is a common disease of dogs all over the world and this is probably why it is one of the diseases puppies are vaccinated against early in life. Tropical countries are very conducive for leptospires to thrive in (Ratnam, 1994; Levett 2001,). The environmental temperature in Nigeria which ranges from 22^{o}C to 40^{o}C averagely, is ideal for the growth of the organisms. The disease is seasonal with peak incidence occurring during rainy seasons in warm climate regions where rapid dessication would otherwise prevent survival (Levett, 2001; Maskey *et al*., 2006). Leptospirosis is the most common bacterial infection transmitted from animals to humans. It is a neglected tropical disease, the epidemiology and global disease burden of which requires further research (Lau, 2009, WHO, 2003).

The problem of rat infestation in the Nigerian environment and within households was reported by Oluwadare (1982) and Omudu and Ati (2010). Rats are generally maintenance hosts for serovars of the serogroups Icterohaemorrhagiae and Ballum (Bolin, 2000). The presence of rats in the environment and within many households poses a major risk of leptospirosis infection to both animals and humans in Nigeria.

Pathogenic serovars of *Leptospira* species have been isolated from water in tropical regions (Alexander *et al*., 1975). Cases of leptospirosis also follow extensive flooding (Chen, 1985; De Lima *et al*., 1990; Fuortes and Nettleman, 1994; French and Holt, 1989; Oliveira *et al*.,1977; Pan American Health Organization, 1998; Park *et al*.,1989; Simo es *et al*., 1969; W.H.O. 2000; Vanasco *et al*., 2000). Flooding is the most common natural disaster around the world, and is expected to occur with increasing frequency as a result of global climate change and extreme weather events (Patz and Kovats 2002). It results from the interaction of rainfall, surface run-off, evaporation, wind, sea level, catchment size, and local topography. Urbanisation, dams, water management practices, land use, deforestation, and agricultural practices are therefore also relevant because of their impact on the above factors (McMichael *et al*., 2006). Large cities are particularly prone to floods due to the combination of large paved, compacted, or roofed areas that are more impermeable than vegetated land. The problem of water being splashed on pedestrians or road users due to either drainage blockage or non-existence of drainage channels in some parts of Nigeria was reported by Jimoh (2008). According to Strahler and Strahler (1977), rainfall factor is the most fundamental in the emergence of overland flow. Flooding can increase the risk of leptospirosis by changing the interactions between the bacteria, humans, animals, and the environment. Severe flooding has recently emerged as a major driver of epidemics around the world (Easton 1999; Ko *et al*. 1999; Maskey *et al*. 2006; Vanasco *et al*. 2008). The poor drainage system in rat infested areas contaminated with urine and the flood caused by abundance of rain may contribute to the occurrence of leptospirosis in Nigeria as stated by Okewole and Ayoola (2009) that heavy coastal rains promote spirochaete survival in dirty flood waters. Flooding in areas with burst water pipes is an important risk factor as organisms in the contaminated environment may easily get into drinking water of pets and livestock as well as household water. The extent to which infection is transmitted depends on many factors including climate, population

density and the degree of contact between maintenance (rats, mice, raccoons, opossums, dogs, cattle e.t.c.) and accidental hosts (Levett, 2001). In Nigeria, the contaminating vaccination-induced "carrier" state that exposes dogs to new and more pathogenic serovars, use of abattoir-offal as dog foods and the fast urbanization in city clinics that permits more dog-wildlife contacts, which also promotes contact with more pathogenic wild serovars (Okewole and Ayoola, 2009) are risk factors for leptospirosis infection in dogs and other animals.

Agunloye *et al.* (2002) reported a seroprevelence of 16.7% to leptospires in both vaccinated and unvaccinated dogs in Ibadan, Nigeria and the following seroprevalences were obtained: *L. canicola* 27.5% *L. grippotyphosa* 25.5%, *L. icterohaemorrhaiage* 25.5% *L. bratislava* 13.7% *L. ballum* 7.8%.

In Nigeria a total of 32, 45 and 54 valuable adult dogs (including referrals) 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, University of Ibadan (Okewole and Ayoola, 2009).

Leptospirosis is a disease that affects almost all animals and any serovar may affect any animal species. There is also no cross protection against the various serovars, so vaccination against one does not necessarily protect against another (Rakesh *et al.*, 2009).

Human beings are not exempted from the woes of leptospirosis. Leptospirosis in humans is geographically widespread with an estimated 300-500,000 severe cases each year and fatality rates of up to 30% (WHO, 2003). The case fatality rate for the severe forms of leptospirosis in humans as reported by Adler *et al*. (1980, 1981, 1982), and Alexander and Rule (1986) was 5 – 40%. Dogs are a significant reservoir for human infection in many tropical countries (Barkin, 1973; Everard *et al*. 1987; Weekes *et al*., 1997; Zavitsanou and Babatsikou, 2008) and may be an important source of outbreak. However, there is no hospital in Nigeria that carries out routine diagnosis of leptospirosis. Consequently, all likely cases are generalized as pyrexia of unknown origin. Leptospirosis has been reported as being responsible for cases of abortion and death in humans (WHO, 2003), moreover, very little information is available on human

leptospirosis in Nigeria although several workers have reported high infection rates in man (Diallo, 1978; Ezeh *et al.*, 1988; Onyemelukwe, 1993). Infection of goats and cattle in Nigeria has also been reported (Agunloye *et al.*, 1997). It is very important to diagnose leptospirosis early and accurately as early diagnosis and appropriate treatment can prevent fatal outcome (Prabhu *et al*., 2010). Inability to do this has caused death that could result in embarrassment to veterinarians, psychosocial problems and economic loss for pet owners and confusion for researchers.

Problem Statement: From the foregoing, the wide host-range, geographical distribution of leptospires, the large number of serovars that cut across various species of animals, including humans, the lack of cross protection among serovars, in addition to the risk factors to the infection in Nigeria, make leptospirosis a very important and interesting disease. Timely diagnosis relies on an effective laboratory test, since the presentation of early–phase leptospirosis is often non-specific (Adler *et al*., 1982, Alexander *et al*., 1963; Alexander and Rule, 1986). Lack of laboratory support and trained laboratory manpower is an important issue in leptospirosis surveillance and control. For isolation of *Leptospira*, selection of the right specimens and tests as well as the correct interpretation of test results are important (Vijayachari and Sehgal, 2006).

1.3 Project Objectives

This project is designed to accomplish the following objectives:

To obtain information on the morbidity, case fatality rates and pattern of occurrence of leptospirosis in dogs within Ibadan, Nigeria.

To determine and compare the accuracy, sensitivity and specificity of Dark Field Microscopy (DFM), Fluorescent Antibody Staining (FAS) and Polymerase Chain Reaction (PCR) for detecting leptospires in cattle, dog and wildlife samples.

To evaluate and compare the rapidity of the three diagnostic techniques.

To evaluate and compare the cost per unit test of the three diagnostic techniques.

1.4 Justification

In Nigeria, there has been a recent upsurge in the keeping of exotic dogs as pets, especially by the middle class (Oluwayelu *et al.,* 2011). This has led to a growing awareness among pet dog owners of the need for veterinary care for their dogs and the establishment of close bonds between these dogs and their owners. Similarly, Nigerian village dogs which are mostly used for hunting purposes enjoy a close relationship with the hunters (Oluwayelu *et al.,* 2011). Consequently, there has also been an upsurge in the number of dog cases tentatively diagnosed as leptospirosis in recent times which could be as a result of increased environmental filth causing the population of reservoir hosts like rats to increase, leading to increase in their rate of contact with domestic animals. Climatic changes, heavy rainfall and poor drainage systems have also resulted in terrible floods which might have contributed to the rapid spread of leptospirosis in Nigeria. An adequate diagnostic technique should therefore be put in place to enable thorough investigation and evaluation of the occurrence of leptospirosis with a view to providing comprehensive baseline epidemiological data on the disease. Routine early and rapid diagnosis of leptospirosis is currently not available in Nigeria, despite its importance in ensuring a favourable clinical outcome of the disease. Diagnosis of leptospirosis should be affordable so that people from all strata of the social class, especially in developing countries would benefit from it. Under-diagnosis and under-reporting of the disease is frequent due to asymptomatic infection and the wide range of symptoms (Center for Food Security and Public Health, 2005). The availability of an adequate diagnostic technique would solve these problems. Accurate diagnosis of leptospirosis would prevent drug (antibiotic) abuse and resistance in veterinary and human practices.

Hypotheses

There is perfect agreement between the results obtained by Dark Field Microscopy and those obtained by Fluorescent Antibody Staining for the diagnosis of leptospirosis.

There is perfect agreement between the results obtained by Dark Feld Microscopy and those obtained by Polymerase Chain Reaction for the diagnosis of leptospirosis.

There is perfect agreement between the results obtained by Fluorescent Antibody Staining and those obtained by Polymerase Chain Reaction for the diagnosis of leptospirosis.

Dark Field Microscopy is not as rapid as Fluorescent Antibody Staining for leptospirosis diagnosis.

Dark Field microscopy is not as rapid as Polymerase Chain Reaction for leptospirosis diagnosis.

Fluorescent Antibody Staining is not as rapid as Polymerase Chain Reaction for leptospirosis diagnosis.

Dark Field Microscopy costs less per unit test than either Fluorescent Antibody Staining or Polymerase Chain Reaction for leptospirosis diagnosis.

CHAPTER TWO

LITERATURE REVIEW

2.1 The genus *Leptospira*

Leptospira species are bacteria of the order Spirochaetales, family Leptospiraceae and genus *Leptospira* made up of about 250 serovars, 7 species and about 25 serogroups. (Faine *et al.*, 1999).

2.1.1 Taxonomy

Leptospira together with the genera *Leptonema* and *Turneria*, is a member of the family Leptospiraceae. *Leptospira* is divided into 20 species based on DNA hybriidzation studies (Adler *et al*., 1982). It is divided into 3 groups on the basis of pathogenicity as: Pathogenic Leptospira: *L. interrogans, L. kirschneri, L. noguchii, L. alexanderi, L. weileii, L. genomospecies 1, L. borgpetersenii, L. santarosai, L. kmety* (Adler and Faine, 1978).

Intermediates or opportunistic Leptospira*: L. inadai, L. fanei, L. broomii, L. licerasiae, L. wolffii* (Adler and Faine, 1977, Adler *et al*., 1986).

Non-pathogenic *Leptospira*: *L. biflexa, L. meyeri, L. wolbachii, L.* genomospecies 3, *L. genomospecies* 4, *L. genomospecies* 5.

Members of the genus *Leptospira* are also grouped into serovars according to their antigenic relatedness. There are currently over 200 recognized serovars. Some serovars are found in more than one species of *Leptospira*. The approved way of writing the nomenclature when including the serovar is to italicize the genus and species, with the genus having an upper case first letter, but the serovar is not italiczed and it is given an upper case first letter e.g. *Leptospira interrogans* serovar Australis, *Leptospira biflexa* serovar Patoc.

2.1.2 Morphology

All members of the genus *Leptospira* have similar morphology. *Leptospira* are spiral shaped bacteria that are 6-20µm long. They are the thinnest of the spirochaetes, 0.1µm in diameter with a wavelength of 0.5µm (Alexander *et al*., 1952). One or both ends of the organism are usually hooked. The number of coils or spirals is usually 18 or more. All leptospires have a common anatomic component made up of an outer envelope or sheath (OS). A helical shaped protoplasmic cylinder (PC) is located within the sheath. Two periplasmic flagella, also referred to as axial filaments, endoflagella and periplasmic fibrils are attached sub terminally to the cell ends between OS and PC (Krieg and Holt, 1984; Hovind-Hougen, 1986).

2.1.2.1 Outer membrane or sheath

Electron microscopy shows that the outer membrane is a three-layered (Nauman, *et al*., 1969, Krieg and Holt, 1984) to five-layered (Anderson and Johnson, 1968; Zeigler and Eseltine, 1975) membrane. The chemical composition of the outer sheath is phospholipids, protein and carbohydrate (Zeigler and van Eseltine, 1975; Auran *et al*., 1972). The outer membrane of leptospire is immunogenic in hamsters (Nunes-Edwards *et al*.,1985; Ribeiro *et al*.,1992). Cinco *et al.* (1988) suggested that the outer membrane contained the leptospiral lipopolysaccharide which has been shown to be serogroup specific (Vinh *et al.*, 1986; Ribeiro *et al.*, 1992). This membrane is also the primary target for antibody – complement mediated bactericidal action (Anderson and Johnson, 1968).

2.1.2.2 Periplasmic flagella (Axial filament)

Two periplasmic flagella are located underneath the outer membrane of the leptospire. Each flagellum is inserted at each end of the leptospire. The flagella of leptospires do not overlap (Krieg and Holt, 1984; Hovind-Hougen, 1986) unlike the flagella of other spirochetes which overlap at the center (Johnson, 1977). Each flagellum consists of a shaft, the hook and the basal body. The flagella of the leptospires are responsible for their motility (Johnson, 1977; Holt, 1978; Hovind-Hougen, 1986; Goldstein and Charon, 1988).The flagella are used for both translational and non translational forms of

movements. Although leptospires give the impression that they are rapidly spinning (Inada *et al.,* 1916), video microscopy indicates that the cell body does not roll, rather the cells are moving so rapidly they give the illusion that the body is spinning (Goldstein and Charon, 1988).

2.2 Adaptive Characteristics

2.2.1 Complement evasion

In contrast to the saprophytic *L. biflexa* which is killed within a few minutes in the presence of normal human serum in vitro, pathogenic *Leptospira* species are able to survive. The latter are more resistant to the action of complement system, especially if they are virulent (Anderson *et al*., 1978; Andreescu *et a1*., 1988).

2.2.2 Immune evasion strategies involved in renal colonization

Infection with host–adapted leptospiral serovars can result in lifelong renal carriage and urinary shedding (Haake *et al*., 2004). Leptospires colonize the proximal renal tubules of reservoir animals, where they are able to replicate and persist, being constantly eliminated in the urine. A wide range of mechanisms probably involved in the ability of leptospires to survive in the kidneys have been suggested (Buckland and Stuart, 1945). *Leptospira* LPS recovered from the rat kidney presents a higher content of the O antigen compared with the LPS of leptospires isolated from guinea-pig liver with acute infection (Bulach *et al.,* 2000). This increased content of LPS O antigen in chronically infected kidneys could constitute an immune evasion strategy. Besides increased LPS O antigen content, proteomic analysis revealed a reduced expression of antigenic proteins in leptospires from rat kidneys in contrast to in vitro cultured bacteria (Burth *et al*., 1997). This antigenic reduction could also reflect a means of escaping from host immune responses.

2.2.3 Biofilm formation

Saprophytic and pathogenic leptospires are able to form biofilms, helping them to survive in environmental habitats and to colonize the hosts (Butler and Endara, 2000). Biofilms

can constitute a barrier against the immune effector cells and molecules, including antibodies and complement. This represents one of the major mechanisms of *Pseudomonas aeruginosa* persistence in chronic infections (Cacciapouti *et al*., 1987). Investigations on biofilm formation by leptospires in renal tubule cells from resistant and susceptible hosts could certainly contribute to the understanding of immune evasion strategies and disease pathology.

2.3 Leptospirosis

Leptospirosis has several synonyms. In cattle, it is referred to as 'infectious haemoglobinuria', 'red water of calves', 'infectious abortion'. In man, leptospirosis is referred to as 'Weil's disease', 'seven day fever', autumn fever', 'swamp or mud fever', and 'fort bragg fever'. In dogs, it is 'yellows' and Stuttgart disease' (Alston and Broom, 1958).

Adolf Weil first described leptospirosis in man in Japan in 1886. He reported an infectious illness manifested as jaundice, nephritis and 'spleen tumour'. Since then the disease has been called Weil's disease in man. The causal organism was not isolated until 1915 when spirochetes were found in infectious jaundice in Japan by Inada and Ido (1915) who named the organism *Spirochaeta icterohaemorrhagiae*. About the same time, Uhlenhurt and Fromme (1915) in Germany described a spirochete which was named *Leptospira icterogenes*. Noguchi (1917) later renamed the organism *Leptospira icterohaemorrhagiae*. In 1917, *L. hebdomadis* was discovered as the causative agent of "seven day fever" in Japan. In 1923, *L. pyrogenes* and *L. bataviae* were first described in Indonesia where clinicians described it as causing 'short term spirochetal fever' (Gsell, 1984). Several other serovars numbering over 200 have been described in various parts of the world including Nigeria.

2.4 Diagnosis

2.4.1 Microscopy

2.4.1.1 Dark field microscopy (DFM).

Dark-field microscope allows a viewer to observe living unstained cells and organisms by simply changing the way in which they are illuminated. Only light that has been reflected or refracted by the specimen forms an image. The field surrounding the specimen appears black, while the object itself is brightly illuminated. Dark field microscopy of body fluids such as blood, urine and cerebrospinal fluid as well as dialysate fluid has been used, but it is reported to have low sensitivity and specificity. Approximately 10^4 leptospires /ml are necessary for one cell per field to be visible by this method (Turner, 1970). Microscopy of blood is of value only during the first few days of the acute illness while leptospiraemia occurs. Leptospires were detected by DFM in some test cases as early as four days prior to the development of symptoms (Alston and Broom, 1958). In another report, all the positive samples were taken not more than six days after onset of symptoms (Wolff, 1954). Most authorities agree that there are too few leptospires in cerebrospinal fluid (CSF) for detection by DFM (Wolff, 1954; Alston and Broom, 1958). It is also believed that direct Dark Field Microscopy of blood is subject to misinterpretation of fibrin or protein threads which may show Brownian motion (Uhlenhuth and Fromme, 1915; Wolff, 1954; and Faine *et al.*, 1999). The dark field microscopy is easy to perform, highly reproducible and does not require special equipment or refrigeration.

2.4.1.2. Fluorescent antibody staining.

The sensitivity of direct microscopic examination may be increased by staining methods. Fluorescent staining of antibody in urine and of cultures is a fast and accurate diagnostic method for detecting the presence of leptospirae and for identifying serotypes (Hodges and Ekdahl, 1973). This test may be used with fresh or frozen tissues or urine and it aids in the discrimination of leptospires against artefacts (Bolin *et al.*, 1989). The Fluorescent antibody staining is very useful for demonstrating leptospires in tissues from animals

which have died of leptospirosis (Cook *et al.,* 1972; Kirkbride and Halley, 1982). It was reported by Smith *et al.* (1966) to be superior to culture and histopathological methods in demonstrating the presence of leptospires in autolysed materials. Reports have also been given of immunofluorscence staining of urine, water and soil (Bolin *et al*., 1989; Hodges and Ekdahl, 1973). Immunofluorescence microscopy is used extensively to demonstrate leptospires in veterinary specimens (Ellis *et al*., 1982).

2.4.1.3 Histopathology

A variety of histopathological stains have been applied to detect leptospires in tissues.

These include silver staining (Stimson 1907) and Warthin-Starry staining for histological examination.

2.4.2 Bacteriology

2.4.2.1 Culture

Various culture media had been used in earlier times for the culture of leptospires (Noguchi, 1912; Johnson and Gary, 1963). However, it was established by Ellinghausen and McCullough (1965) that leptospires would grow in a medium containing long-chain fatty acids as a nutritional source provided that serum albumin was provided as detoxicant.

Culture is rarely used in the clinical setting because it is very tedious, complicated, expensive, technically demanding, time consuming, requires prolonged incubation and has low sensitivity. The organism has a relatively long doubling time (6-8hrs). It is highly infectious and could be hazardous to the laboratory workers. However, this technique has an important role in the study of outbreaks and global epidemiology and provides a crucial pool of clinical strains for studies of pathogenesis (Wiuthiekanun *et al*., 2007).

2.4.2.2 Inoculation of laboratory / experimental animals.

Virulent leptospires will cause an infection in suitable laboratory animals, and these can be used for primary isolation of leptospires from clinical or environmental specimens (Faine *et al.*, 1999). According to these authors, hamsters, gerbils and guinea-pigs are most often used. It is important to check the animals before use to ensure they are not carriers of leptospires (Yukawa *et al.*, 1990). The route of infection is usually by intra-peritoneal injection, although deposition of leptospires on the conjuctival surface is also highly effective. Direct dark-field examination of urine proved superior to guinea-pig inoculation as a method of detecting leptospiruria and it was suggested that the former technique could be adopted with an advantage as a routine aid to diagnosis (Mitchell *et al.*, 1966).

2.4.3 Serology

2.4.3.1 Microscopic Agglutination Test (MAT)

This is the reference method for serological diagnosis of leptospirosis and it involves a reaction between the patient's sera and live antigen suspensions of leptospiral serovars. The MAT is a complex test to control, perform and interpret (Turner, 1968). Live cultures of all serovars required for use as antigens must be maintained. The repeated weekly subculture of large numbers of strains presents hazards for laboratory workers and laboratory acquired infections have been reported (Alexander *et al*., 1952; Pike, 1976). Other drawbacks include the continuous risk of cross-contamination of the antigen cultures necessitating periodic verification of each serovar. MAT titres are affected by the culture medium in which the antigens are grown (Myers, 1976). The MAT is read by dark-field microscopy. The end point is the highest dilution of serum at which 50% agglutination occurs. This is difficult to detect, so 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. Antibodies are not formed in the patients until after 7-10 days from the onset of symptoms. This makes early diagnosis and prompt treatment impossible. There is also the limitation of accurate pathogen identification due

to the high degree of cross-reactivity between leptospires as well as the difficulty in distinguishing antibody titers of a chronically infected animal from titers stimulated by vaccination.

2.4.3.2 Other serological tests

Other serological tests such as Complement Fixation Test (CFT) (Koury *et al*., 1991, Enzyme Linked Immuno-Sorbent Assay (ELISA) (Hartman *et al*., 1984, L-Ard *et al.*, 2011) Indirect Haemaglutination assay (IHA) (Effler *et al.,* 2002) among others have also been used for detecting leptospires.

2.4.4 Polymerase Chain Reaction (PCR)

This involves the amplification of specific fragments of leptospiral genomic DNA in clinical samples. It requires the selection of specific primers to allow amplification of all strains that are classified as pathogenic or potentially pathogenic (Faine *et al*., 1999). According to Gravekampe *et al.,* (1993), red blood cells inhibit PCR. The maximum volume which could be processed without inhibition of the PCR is 100µl. However, 1-3 ml of serum could be used without any problem. The specificity of the PCR assay can be adjusted by the choice of primers (Van Eys *et al.*, 1989). One of the major advantages of the PCR is the fact that it is able to detect small numbers of organisms in clinical samples.

2.5 Leptospirosis in dogs

2.5.1 History

The first case of leptospirosis identified in dogs was caused by *L. icterohaemorrhagiae* as reported by Okell *et al.* (1925). These workers showed that the disease could exist in three forms: a hyper-acute fulminating disease with death occurring within one or two days; an illness with jaundice as the most dramatic clinical feature – the typical canine " yellows" or simply a slight fever with transient gastrointestinal upset. Detection of another variety of canine leptospiral disease with mainly nephritic feature was ascribed to

the Dutch workers Klarenbeek and Schuffner (1933). The disease was caused by *L. canicola.*

2.5.2 Aetiology

The serovars mainly involved in canine leptospirosis in Canada are no longer Canicola and Icterohaemorrhagiae, as reported before the 1970s ; they now include Grippotyphosa and Pomona as the most common serovars (Prescott *et al*.,1991), although Bratislava (Nielsen *et al*.,1991) and possibly Autumnalis are sometimes the infecting serovars. The reason for the increase of leptospirosis in dogs and the change in the serovars involved may be the increased and endemic infection of urban wildlife (notably raccoons, skunks) with leptospirosis, combined with increased numbers of urban wildlife and an increasing index of suspicion by veterinarians, thus promoting serological testing, as well as successful control by vaccination of the previously important serovars (Prescott *et al.,* 2002).

Cases of canine leptospirosis have been reported in Nigeria. Studies carried out on dog population in a part of Ibadan, Nigeria revealed that 68% of the household dogs were outdoor, scavenging most of the time (Faleke, 2003). Antibody tests against *Leptospira* organisms in Nigeria using modified Microscopic Agglutination Test showed that 11.4% of pet dogs and 12.2% of stray dogs were positive (Ogunkoya *et al*., 1990). A study on the serological reactions of vaccinated and unvaccinated dogs in Ibadan, Nigeria to eight leptospiral serovars was reported by Agunloye *et al.* (2002). The study showed that overall seroprevalence to leptospires was 16.7% while in unvaccinated dogs it was 14.4%. Significantly more vaccinated dogs, than unvaccinated dogs were affected. The following seroprevalences were obtained: *L. canicola* 27.5% *L. grippotyphosa* 25.5%, *L. icterohaemorrhaiage* 25.5% *L. bratislava* 13.7% *L. ballum* 7.8% (Agunloye *et al*., 2002). Vaccination-induced carrier state has been reported to be responsible for the introduction of new serovars into the Nigerian environment. This has contributed to the introduction and spread of new strains of *Leptospira* other than the regular serovars, Canicola and Icterohaemorrhagiae into the environment (Okewole and Ayoola, 2009).

2.5.3 Clinical signs

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. 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 a reluctance to move, stiffness, shivering, progressive weakness and depression. Dogs may vomit and/or have diarrhea resulting in rapid dehydration and excessive thirst. Injected mucus membranes are typical, often with widespread petechial and ecchymotic hemorrhages; icterus is uncommon, and it occurs more frequently in dogs infected with *L. icterohemorrhagiae* (McDonough, 2001).

In dogs, the incubation period varies between 3 and 20 days; therefore, this is the relevant period of exposure that must be considered. The most common early signs of disease are anorexia, lethargy, vomiting and fever. Also seen are weight loss, increased drinking and urinating (polydipsia/polyuria), diarrhea, abdominal/lumbar pain, icterus / jaundice, stiffness/reluctance to walk (myalgia), enlarged kidneys (renomegaly), small areas of hemorrhage (petechia) or sometimes severe hemorrhage, and low platelet count (thrombocytopenia) in some of the affected cases (Prescott *et al.,* 2002).

2.5.4 Economic importance and Public health significance

Humans become infected through contact with water, food or soil containing urine from these infected animals. This may happen by swallowing contaminated food or water or through skin contact. It could also be spread by splashing contaminated water to the mouth or eyes or by exposing open wounds to contaminated water. Pet owners could also be exposed to the leptospires infecting their pets because humans are incidental hosts.

2.5.5 Vaccination

2.5.5.1 Vaccination and control

There are many leptospiral serovars but there is no cross protection among serovars. The following serovars cause infection in dogs: *L. icterohaemorrhagiae, L. canicola, L. grippotyphosa, L. pomona , L. autumnalis and L. bratislava.* Vaccines are not available

against the last two. Vaccination may protect against clinical disease but it does not prevent the dog from being a carrier. Immunity conferred by leptospiral vaccine is temporary, and vaccination has to be repeated as the immunity wanes. Moreover, immunized dogs may be infected with serovars other than those contained in commercial vaccines (Cole *et al.,* 1982; Everard *et al*., 1987; Prescott *et al*., 1991; Brown *et al*,. 1996; Okewole and Ayoola, 2009). A vaccine has been released recently which includes serovars Grippotyphosa and Pomona (in addition to the traditional vaccine strains) in response to the increasing incidence of canine infection with these serovars.

2.6 Leptospirosis in cattle

2.6.1 History

Bovine leptospirosis was first described in the USSR by Michin and Asinov (1935) under the name infectious haemoglonuria. Since then leptospirosis in cattle has been reported in several countries of the world (Amatredjo and Campbell, 1975).

2.6.2 Aetiology

The species of leptospires associated with bovine leptospirosis was *L. interrogans* . Several serovars are involved, among which are *: L. interrogans* serovar Pomona, *L. interrogans* Hardjo, *L. interrogans* serovar Grippotyphosa, *L. interrogans* serovar Australis, *L. interrogans* serovar Icterohaemorrhagiae, and *L. interrogans* serovar Canicola depending on the most prevalent serovars in an environment. Leptospirosis caused by *L. interrogans* serovar Hardjo is a major cause of abortion in cattle (Hathaway and Little, 1983). Previous studies on the clinical, serological and bacteriological detection of leptospires in cattle had been reported in Nigeria (Agunloye *et al*., 1997; Agunloye *et al*., 2000).

2.6.3 Clinical signs

Infection with serovar Hardjo usually results in no or mild acute clinical signs but produces a renal carrier state associated with long term urinary shedding. Clinical signs of serovar Hardjo infection in dairy cattle are subtle and often involve decreased

reproductive efficiency and milk production (Bolin, 2003). Abortions, still births or birth of weak calves are seen in serovar Hardjo infections when cows are infected for the first time when pregnant. Infected but apparently healthy calves could be born, followed by retention of foetal membrane. Abortions caused by serovar Hardjo are usually sporadic. Herds contracting the infection for the first time may have abortion in as high as 30% of the animals (Ellis, 1994) while in herds with endemic infection, abortion may be in just about 5% of the animals (Anon, 1986)

2.6.4 Economic significance and public health importance

Losses due to leptospirosis infection in a herd include:

Calf deaths, abortion, reduced milk production, treatment costs, increased culling rate employment costs for relief staff when workers become infected, workers compensation costs and legal liability for preventable disease contracted by staff at work (Radostits *et al*., 1994; Turner and Stephens, 2006).

Approximately 75% of UK cattle have been exposed to *Leptospira hardjo*, for which they are the 'maintenance host', which means that after infection they harbour the bacteria in their kidneys for months, even years, excreting many leptospires in their urine so acting as a reservoir of infection for other cattle. Both dairy and suckling cattle can be affected (Owen, 2010). The overall costs of disease caused by leptospirosis average out at £68-£106 /cow in an affected herd. In terms of cost per litre of milk, this works out at a loss of 0.91-1.41 pounds per litre (Owen, 2010). Leptospirosis induced abortion rate of 1-18% was reported by Pritchard (1986) in a study of 50 recently infected herds in England. The cost of a single leptospiral infection was put at £500.00 (Bennett *et al*., 1999). The authors claimed that in addition to the reduced milk yield, infertility could also result from *L. hardjo* infections. In 1955, Morse estimated an annual loss of $100 million to the livestock farmer due to Pomona infection in cattle. Miller *et al.,* (1991) reported that two percent of adult cattle in the USA are renal carriers of leptospires. Little or no work has been done on the economic effects of bovine leptospirosis in Nigeria.

Humans working with cattle can also be “incidental hosts” for *L. hardjo* which causes a disease that may go unrecognized because of its flu-like symptoms. About 25 cases of leptospirosis are recorded by the Public Health Laboratory every year (Owen, 2010).

2.6.5 Vaccination

Immunity to leptospirosis is largely humoral (Adler and Faine, 1977) and is relatively serovar specific. Thus, immunization protects against disease caused by the homologous serovar or antigenically similar serovars only. Vaccines must therefore contain serovars representative of those present in the population to be immunized. Immunization has been widely used for many years as a means of inducing immunity in animals and humans, with limited success. Early vaccines were composed of suspensions of killed leptospires cultured in serum-containing medium, and side effects were common. Modern vaccines prepared using protein-free medium are generally without such adverse effects (Bey and Johnson, 1978; Christopher *et al* 1982). In developed countries, pigs and cattle are widely immunized, as are domestic dogs, but in most developing countries, vaccines which contain the locally relevant serovars are not available. Most vaccines require booster doses at yearly intervals.

Most bovine and porcine vaccines contain serovars Hardjo and Pomona; in North America, commercial vaccines also contain serovars Canicola, Grippotyphosa, and Icterohaemorrhagiae. Protection against Hardjo infection has been suboptimal, but one vaccine has recently been shown to offer good protection (Bolin *et al.,* 1989).

Canine vaccines generally contain serovars Canicola and Icterohaemorrhagiae. Vaccines protect against disease and renal shedding under experimental conditions (Broughton and Scarnell, 1985), but transmission of serovar Icterohaemorrhagiae from immunized dogs to humans has been reported (Feigin *et al*., 1973).

Human vaccines have not been applied widely in Western countries. Immunization with polyvalent vaccines has been practiced in the Far East, where large numbers of cases occur in ricefield workers, such as in China (Chen, 1985) and Japan. In France, a monovalent vaccine containing only serovar Icterohaemorrhagiae is licensed for human

use. A vaccine containing serovars Canicola, Icterohaemorrhagiae, and Pomona has been developed recently in Cuba (Martinez *et al*. 1998). A vaccination program can be a very cost-effective management tool in avoiding the potentially high production losses, animal suffering and the risk of human infection due to leptospirosis in a dairy herd.

A vaccination program can provide long-term immunity in cattle against leptospirosis. The aim is to vaccinate all susceptible cattle before infection occurs, so that chronic urinary shedding is prevented. When leptospirosis is already present in the herd the following points are important:

Vaccination will immunize young uninfected cattle against the organism.

The older chronic carriers will be gradually culled, leaving only immune cattle.

Vaccination does not prevent urinary shedding of the organism in previously infected cattle.

Veterinary treatment is required to cure the disease.

When the disease is not already present:

A vaccination program will prevent infection;

Consequently urinary shedding of bacteria will not occur; and

The herd will remain free of leptospirosis with an effective vaccination program (Turner and Stephens, 2006).

2.6.5.1 Vaccination program

Vaccinate all cattle with a combined *hardjo* and *pomona* (‘2-in-1’) vaccine.

The first dose is given at three to six months of age.

A second dose should be given four to six weeks later.

Calves vaccinated before six months of age (to protect against *pomona redwater*) require revaccinating at six months, and again four to six weeks later, as maternal antibodies may interfere with acquiring long-term immunity before six months of age.

Cattle should be given a booster vaccination every 6 to 12 months as required.

Boosters are best given two–four weeks before calving or before the wet season.

Six-monthly vaccination may be warranted, if a farm has a history of leptospiral problems.

Vaccinate all livestock on the property as all stock, including bulls and steers, can be infected and become chronic urinary shedders.

All new animals brought onto a property should be fully vaccinated before being introduced into the herd.

It is important to follow the advice given for the specific vaccine being used as recommendations vary for different manufacturers.

Using a combined leptospirosis-clostridial ('7-in-1') vaccine will improve the efficiency and economy of the vaccination program.

2.6.5.2 Vaccination costs

The cost to vaccinate a herd can vary significantly, as the price per head can be affected by:

the total number of cattle to be vaccinated — larger herds generally cost less per head;

the regional prices;

the price from the particular supplier of the vaccine.

It is important to compare the different brands and various retailers to ensure implementing the most economic vaccination program.

2.6.5.3 Important considerations when vaccinating cattle

The manufacturer's instructions should be followed closely.

Store and handle vaccines correctly to ensure their effectiveness is not reduced.

Safety precautions for workers handling vaccines and associated equipment should be adhered to carefully.

Ensure safe disposal of used equipment, avoiding environmental contamination.

Animals should be in good health to optimize the immunity gained. Vaccination does not provide instant protection — generally full protection doesn't occur until up to four weeks after the initial doses.

2.7 Leptospirosis in wildlife

Each species of wildlife has serovars of *Leptospira* that live in relative harmony with it. Such wildlife are referred to as 'primary reservoir hosts'. Leptospires become adapted to these 'primary reservoir' hosts. Rats are the primary reservoir hosts for the pathogenic leptospiral species associated with the severe forms of human leptospirosis (Bonilla-Santiago and Nally, 2011). Animal reservoirs that may pose a risk for human exposure include dogs, rats, raccoons, skunks and livestock (Centers for Disease Control and Prevention, 2005). Some parts of the world have observed leptospirosis in their wildlife populations. For example, Fennestad and Borgpetersen (1972) found varying frequencies of different serovars in small wild mammals (ranging from 0.4 to 58%) and deer (4%) in Denmark.

2.7.1 Public health importance

A resurgence of leptospirosis in dogs reported in some parts of North America is said to be due to exposure of pets to increased population of urban wildlife with a shift in prevalence of serovars from Canicola and Icterohaemorrhagiae to Grippotyphosa and Pomona (Prescott, 2002). The primary reservoirs of Grippotyphosa and Pomona are skunks, opossums and raccoons (North American Veterinary Conference, 2005). A very

similar shift in prevalence of serovars in Nigeria was reported by Okewole and Ayoola (2009). They stated that new non-vaccinal serovars of lence than the old vaccinal serovars of Canicola and Icterohaemorrhagiae due to growing pet-wildlife contact in urbanization that exposes the pets to these former wildlife serovars.

2.8 Leptospirosis in humans

In developing countries leptospirosis is usually a disease associated with sugar-cane workers, farmers and military troops exposed to soil or water contaminated by the urine of infected wild or domestic animals. Other occupational groups may acquire disease by direct contact with animal urine (e.g. veterinarians, workers in animal husbandry and pet shop employees, miners, rodent control workers, fish farmers, rice field workers, banana farmers) and are therefore at risk (Chan *et al.*, 1989; Padre *et al*., 1988; Campagnolo *et al*., 2000; Terry *et al*., 2000). Leptospirosis has been associated with flooding and residents in inner cities where there is contact with rodent and dog urine. It has also emerged as recreational water hazard in temperate and tropical zones, particularly for adventure racers, swimmers and those involved in kayaking and water skiing (Wong, 1977; Travejo *et al*., 1998; Vinetz, 1996; 2001; Demers, 1983; Sevjar, 2003; ProMED-mail, 2004).

2.8.1 Aetiology and risk factors

Many sporadic cases of leptospirosis are associated with activities of daily life; specifically, many cases result from barefooted walking in damp environment or gardening with bare hands (Douglin *et al.*, 1997, Everard *et al*., 1992)

A case control study has shown dog ownership and the presence of rodents to be a risk factor for leptospirosis in the context of flooding in a developing country (Trevejo *et al.*, 1998). Splashes of infected material into eyes, ingestion of contaminated food or water , inhalation of aerosols of contaminated fluid are all exposures that pose a risk of transmission (Jorge, 1932; Public Health Agency of Canada, 2001). Leptospires may also be able to penetrate intact skin that has been in water for a long time (Center for Food

Security and Public Health, 2005). Normal interaction with an infected pet is considered a low risk for infection. Assisting with the birth of a new-born from an infected animal may be a high-risk activity (New Brunswick Health and Wellness, 2002; Centers for Disease Control and Prevention, 2005). Milking of cows also poses a risk of infection from *Leptospira interrogans* serovar Hardjo and Pomona (Blood and Radostits, 1989). Exposure could occur in brackish tidal pools frequented by pets and their owners along coastal areas where strandings or colonies of these animals occur (Gulland, 1999).

2.8.2 Public health importance

Worldwide, about 20% of cases are said to be associated with pets or rodents in or around the home (Leptospirosis Information Center, 2006). There are few cases of reports of leptospirosis transmitted from pets to humans. However it was not clear whether transmission was associated with illness in the animals (Wong, 1977; ProMED- mail, 2004). A New Zealand survey showed 34% seroprevalence of *Leptospira* among farmers milking cows (Blood and Radostits, 1989). Farmers could prevent occupational exposure by preventing contact of livestock with rodents, wild animals and other livestock, which potentially could be infected. Farmers should reduce the exposure of livestock to contaminated water by avoiding urine drainage into water sources and draining swampy land.

There is evidence that leptospirosis may be transmitted to infants through breast milk causing infection (Center for Food Security and Public Health, 2005).

2.8.3 Clinical signs

Symptoms are usually fever with sudden onset, chills, headache, severe muscle pain, malaise and conjunctivitis. Other symptoms may include meningitis, gastrointestinal tract symptoms and a rash. The disease is generally biphasic: a leptospiraemic or febrile stage is followed by a convalescent or immune phase. The disease can manifest as a milder anicteric form or a more severe icteric form (5-10% of cases). The case fatality rate is low (1-5%) but may reach 20% in outbreaks of icteric disease associated with hepatorenal failure (Weil's disease) in the absence of dialysis.

Asymptomatic infection also occurs. The severity of the disease varies with the infecting serovar. Clinical illness can last from a few days to several weeks and shedding of the

organism may persist for months and even years. Illness may be more severe in the elderly and may result in foetal loss in pregnant women (Heymann, 2004; Brown and Prescott, 2008). The incubation period in humans is usually ten days with a range of two to thirty days. The period of transmissibility is at its peak during infection. However, prolonged excretion through the urinary tract can occur both in animals and humans (Heymann, 2004).

Late-onset uveitis may result from an autoimmune reaction to subsequent exposure (Ocular manifestations of severe leptospirosis were noted in early reports (Weil, 1886; Weekers and Firket, 1916). Conjunctival suffusion is seen in the majority of patients in some series (Martins *et al*., 1998). 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. Chronic visual disturbance, persisting 20 years or more after the acute illness, has been reported (Shpilberg *et al*.,1990).

The incidence of ocular complications is variable, but this probably reflects the long time scale over which they may occur. In the United States the incidence was estimated at 3% (Heath *et al*., 1965), while in Romania an incidence of 2% was estimated between 1979 and 1985 (Andreescu *et al*., 1988). However, in abattoir workers with evidence of recent leptospirosis, the latter authors reported an incidence of 40% (Andreescu *et al*., 1988).

In most cases uveitis is presumed to be an immune phenomenon, but leptospires have been isolated from human and equine eyes (Alexander *et al*., 1952; 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; Faine, 1994).

Recently, a large cluster of cases of uveitis was reported from Madurai in southern India following an outbreak of leptospirosis which occurred after heavy flooding (Rathinam *et al*., 1997; Chu *et al*., 1998). The majority of affected patients were males, with a mean age of 35 years (Rathinam *et al*., 1997). Eyes were involved bilaterally in 38 patients

(52%), and panuveitis was present in 96% of eyes. Other significant ocular findings included anterior chamber cells, vitreous opacities, and vasculitis in the absence of visual deficiency.

Acute infection in pregnancy has been reported to cause abortion (Chung *et al*., 1963) and fetal death (Coghlan and Bain, 1969; Faine *et al*., 1984). In one of the cases reported by Chung *et al.*, (1963), leptospires were isolated from amniotic fluid, placenta, and cord blood; the infant was mildly ill and was discharged at 2 weeks of age. In another case, a neonate developed jaundice and died 2 days after birth (Lindsay and Luke, 1949). Leptospires were demonstrated in the liver and kidneys by silver staining, but serological evidence of leptospiral infection in the mother was only obtained 2 weeks after delivery. Leptospires have been isolated from human breast milk (Chung *et al*., 1963), and in one case serovar Hardjo was probably transmitted from an infected mother to her infant by breast-feeding (Bolin and Koellner, 1988).

Rare complications include cerebrovascular accidents (Lessa and Cortes, 1981; Forwell *et al*., 1984;), rhabdomyolysis (Solbrig *et al*., 1987; Martinelli *et al*., 1994; Coursin *et al*., 2000), thrombotic thrombocytopenic purpura (Laing *et al*., 1990), acute acalculous cholecystitis (Bahaman *et al*., 1988 Monno and Mizushima, 1993; Vilaichone *et al*., 1999), erythema nodosum (Derham,1976), Kawasaki syndrome (Humphrey *et al*., 1977, Wong *et al*., 1977), reactive arthitis (Winter *et al*., 1984), epididymitis (Houghton and Proce, 1986), nerve palsy (Sharma *et al*., 1999; Tong *et al*., 1971), male hypogonadism (Panidis *et al.*, 1994), and Guillain-Barré syndrome (Morgan and Cawich, 1980). Cerebral arteritis, resembling Moyamoya disease, has been reported in a series of patients from China (Ximin *et al*., 1980).

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 described above, 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 Parma *et al*., (1987), Faine (1994) and Lucchesi and Parma (1999), 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.

One follow-up study of 11 patients with a mean time of 22 years (range, 6 to 34 years) after recovery from acute leptospirosis has been reported (Shpilberg *et al*., 1990). Four patients complained of persistent headaches since their acute illness. Two patients complained of visual disturbances; both had evidence of past bilateral anterior uveitis. No biochemical or hematologic abnormalities were detected to suggest continuing liver or renal impairment. No studies to date have attempted to confirm the persistence of leptospires in the tissues of patients who have subsequently died of other causes.

2.8.4 Epidemiology

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 also results in infection via the mucous membranes of the respiratory tract. Rarely, infection may follow animal bites (Silverstein, 1953; Barkin *et al*., 1974; de Souza, 1986; Luzzi *et al*., 1987; Gollop *et al.,* 1994). Direct transmission between humans has been demonstrated rarely. However, excretion of leptospires in human urine months after

recovery has been recorded (Johnson, 1950; 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 (Doeleman, 1932; 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 avocational exposures. Occupation is a significant risk factor for humans (Waitkins, 1986). Direct contact with infected animals accounts for most infections in farmers, veterinarians, abattoir workers (Chan *et al*., 1989; Campagnolo *et al*., 2000; Terry *et al*., 2000), meat inspectors (Blackmore *et al*., 1979), rodent control workers (Demers *et al*., 1985), and

other occupations which require contact with animals (Anderson *et al*., 1978; Looke, 1986). Indirect contact is important for sewer workers, miners, soldiers (Buckland and Stuart, 1945; Mackenzie *et al.,* 1966; Johnston *et al*., 1983), septic tank cleaners, fish farmers (Robertson *et al.,* 1981; Gill *et al.,* 1985), gamekeepers, canal workers (Andre-Fontaine *et al*., 1992), rice field workers (Wang, 1965; Famatiga *et al*., 1972; Padre *et al.,* 1988), banana farmers (Smythe *et al.,* 2000); and sugar cane cutters (Cotter, 1936).

Miners were the first occupational risk group to be recognized (Inada *et al*., 1916). The occurrence of Weil's disease in sewer workers was first reported in the 1930s (Fairley, 1934; Alston, 1935; Johnson *et al*., 1937; Stuart, 1939). Serovar Icterohaemorrhagiae was isolated by guinea pig inoculation from patients, from rats trapped in sewers (Alston, 1935; Johnson *et al.,* 1937), and from the slime lining the sewers (Alston, 1935). 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 (Chang *et al*., 1948).

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 occurred 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, 1986).

A serological examination of sera from 661 human volunteers in various occupations comprising 248 coal miners, 138 butchers and abattoir workers, 213 local farmers and 62 hospital laboratory personnel from various communities in Enugu and environs of eastern Nigeria was undertaken between January 1990 and March 1991 (Onyemelukwe, 1993). According to this report, leptospiral antibody titres of 1:100 and above were present in 89

(13.5%). The highest prevalence of antibodies to individual serovars were canicola 21 (23.6%), hardjo 17 (19.1%), pomona 13 (14.6%), icterohaemorrhagiae 11 (12.4%), pyrogenes 8 (9.0%), autumnalis 8 (9.0%) and grippotyphosa 7 (7.9%). Among the occupational groups examined, the coal miners were particularly at risk with a prevalence rate of 41 (46%), followed by the butchers/abattoir workers 26 (29.2%), farmers 18 (20.2%) and hospital laboratory personnel 4 (4.5%).

Fish farmers have also been shown to be at risk (Robertson *et al*., 1981), particularly for infection with serovars of serogroup Icterohaemorrhagiae (Gill *et al.,* 1985), 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*., 1985).

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 (Crawford and Miles, 1980; Blackmore and Schollum 1982; Waitkins, 1986; Padre *et al*,. 1988; Sakula and Moore, 1969; Bercovich *et al*., 1990), in particular with milking of dairy cattle (Hart *et al*., 1984; Skilbeck and Miller, 1986; Levine, 1984). Human cases can be associated with clinical disease in cattle (Hart *et al.,* 1984; Sakula and Moore, 1969). Cattle are maintenance hosts of serovar Hardjo (Ellis *et al*., 2000), and infection with this serovar occurs throughout the world (Myers and Jelambi, 1975; Bahaman, 1988; Prescott *et al*., 1988). Many animals are seronegative carriers (Thiermann, 1983; Hathaway *et al*., 1986; Ellis *et al.,* 2000). After infection, leptospires localize in the kidneys (Orr and Little, 1979; White *et al.,* 1982; Thiermann, 1983; Gregoire *et al*., 1987; Prescott *et al.,* 1987) and are excreted intermittently in the urine (Ellis and Michna, 1977). In a bacteriological survey of leptospirosis in Zaria, Nigeria, leptospires were recovered from 5 of 74 bovine kidneys (Diallo and Dennis (1982). Also, Six leptospiral strains belonging to two serogroups were isolated from 525 bovine kidneys (1.1% of which were positive) obtained from Jos municipal abattoir, Plateau State Nigeria (Ezeh *et al.,* 1989). Five of these belonged to the serogroup Pyrogenes while the sixth one belonged to serovar Hardjo of the Hardjo-bovis group.

Serovar Hardjo causes outbreaks of mastitis (Ellis *et al.,* 1976) and abortion (Ellis and Michna. 1977). Serovar Hardjo is found in aborted fetuses and in premature calves (Ellis *et al.,* 1976; Ellis and Michna, 1976; Hathaway *et al*., 1982; Giles *et al.,* 1983). In addition, serovar 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 (Ellis *et al*., 1985), and the genital tract and urinary tract of more than 50% of cows (Ellis *et al.,* 1986; Ellis and Thiermann, 1986) and bulls (Ellis *et al*., 1986). 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 (Slee *et al.,* 1983; Jerret *et al.,* 1984; Elder *et al.,*1985). In Scotland, 42% of cattle were seropositive for Hardjo, representing 85% of all seropositive animals (Ellis and Michna, 1976). In the United States, serovar Hardjo is the most commonly isolated serovar in cattle (Ellis and Thiermann, 1986), but Pomona also occurs.

There is a significant risk associated with recreational exposures occurring in water sports (Mumford, 1989) including swimming, canoeing (Jevon *et al,*. 1986; Shaw, 1992), white water rafting (Wilkins *et al.,* 1988; van Crevel *et al.,*1994; Reisberg *et al*.,1997), fresh water fishing, and other sports where exposure is common, such as potholing and caving (Waitkins and Buchan, 1987). The potential for exposure of large numbers of individuals occurs during competitive events (Centers for Disease Control and Prevention, 1998; Centers for Disease Control and Prevention, 2000; Communicable Disease Surveillance Centre, 2000; Evans and Baranton, 2000). 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 (Chen, 1985; de Lima *et al.,* 1990; Epstein *et al.,* 1995; French and Holt, 1989; Fuortes and Nettleman, 1994; Oliveira *et al.,* 1977; Pan American Health Organization, 1998; Park *et al*., 1987; Simoes *et al.,* 1969; Vanasco *et al*., 2000; W.H.O., 2000).

Pathogenic serovars have been isolated from water in tropical regions (Alexander *et al*., 1975) and in the United States, where serovars Icterohaemorrhagiae, Dakota, Ballum, Pomona, and Grippotyphosa have been recovered (Crawford *et al.,* 1971; Diesch and

McCullough, 1966; Gillespie and Ryno, 1963). 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 (Chang *et al.,* 1948; Gordon Smith and Turner,1961), but in river water survival is shorter and is prolonged at lower temperatures (Chang *et al.,* 1948; Crawford *et al*., 1971). The presence of domestic sewage decreases the survival time to a matter of hours (Chang *et al*., 1948), 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 (Smith and Self, 1955). When soil was contaminated with urine from infected rats or voles, leptospires survived for approximately 2 weeks (Karaseva *et al.,* 1973; Smith and Self, 1955). 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 avocational exposures that occur during the activities of daily life (Everard *et al*., 1992; Perrocheau and Perolat 1997) like handling plants and soil without wearing protective gears (Douglin *et al*., 1997). 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 (Agrawal and Srivastava, 1986).

Three epidemiological patterns of leptospirosis were defined by Faine (1994). 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 is 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 Control of rodent populations, drainage of wet areas, and occupational hygiene are all necessary for prevention of

human leptospirosis. These are also the areas where large outbreaks of leptospirosis are most likely to occur following floods, hurricanes, or other disasters (Chen, 1985; de Souza, 1986; Epstein *et al*., 1995; French and Holt, 1989; Fuortes and Nettleman, 1994; Oliveira *et al*, 1977; Pan American Health Organization, 1998; Park *et al*,. 1987, Simoes *et al*., 1969; Vanasco *et al*., 2000). 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. This type of infection is now rarely seen in developed countries (Derham, 1976), but is exemplified by the recent rediscovery of urban leptospirosis in Baltimore (Vinetz *et al*, 1996) and by outbreaks occurring in slum areas in developing countries (Ko *et al*,1999).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.

2.8.5 Prevention and Treatment

Understanding the epidemiological / epizootiological features of leptospirosis is a critical step in designing interventions for diminishing the risk of disease transmission. Leptospirosis is better prevented than cured. The following precautionary measures are therefore recommended: Surfaces contaminated with urine or other materials from infected animals should be cleaned with antibacterial cleaning solution, detergent or a solution of one part household bleach and ten parts water (Centers for Disease Control and Prevention, 2005).

People working in animal husbandry should consider wearing appropriate personnel protective equipment such as gloves and boots when touching animals or working in stalls and stables (Centers for Disease Control and Prevention, 2005).

Pet access to food preparation areas, human bedding or garden area where food is grown should be discouraged to avoid contamination. Pet owners should avoid letting their babies or toddlers crawl or play in areas where there may be fresh urine. Pet owners who may have cuts and abrasions should cover them with water proof dressings if their pet is affected.

Pre-exposure prophylaxis may be beneficial for people who will be experiencing exposures that carry a high risk of infection. These include soldiers training in tropical regions, adventure tourists who will have freshwater exposure (especially tropical) and veterinarians who will be working with infected animals. Doxycycline at 200mg once for a single exposure or once a week throughout ongoing exposure is recommended (Guidugli *et al*., 2000; Heymann 2004; Brown and Prescott, 2008,).

Antibiotic post-exposure prophylaxis (e.g. doxycycline at 200 mg) is indicated for persons who have been exposed to leptospires. However, the risk of transmission through normal human contact with animals is considered low if there is no physical contact with the animal's urine (Heymann 2004; Brown *et al.,* 2008). Therefore antibiotic prophylaxis is not routinely offered to protect owners of animals infected with leptospirosis.

Doxycycline prophylaxis is also contra-indicated in pregnant or breast-feeding women and children under the age of eight. As indicated, it may be prudent for these individuals to avoid clean-up of animal wastes and contact with pets during peak periods of transmission (1-3 weeks after onset).

2.8.6 Immunity

Immunity against Leptospira depends on the production of circulating antibodies directed against serovar specific lipopolysaccharides (LPS). Interestingly, leptospiral LPS differs from gram-negative LPS in several biochemical, physical and biological properties (De Souza and Koury, 1992). Although crucial in the early stage of infection, not much is known about the innate immune response to Leptospira. Murine models, using either heat-killed and live bacteria or LPS, showed evidence that both TLR2 and TLR4 play a role (Nahori *et al.,* 2005; Viriyakosol *et al*., 2006). Leptospiral LPS was shown to be recognized by both TLR2 and TLR4 in murine cells (Nahori *et al*., 2005), whereas leptospiral lipoproteins were recognized by TLR2 in murine kidney epithelial cells. Mice with combined TLR2/TLR4 deficiency were found to be highly susceptible to lethal leptospirosis (Nally *et al*, 2005; Viriyakosol *et al.,* 2006; Chassin *et al*., 2009).

CHAPTER THREE

3.0 RETROSPECTIVE STUDY

3.1 INTRODUCTION

A retrospective study looks backward in time. It uses existing data that have been recorded for reasons other than research. It could be referred to as 'chart review' (Hess, 2004). According to this author, a particularly useful application of a retrospective study is a pilot study that is completed in anticipation of a prospective study. The retrospective study can help to focus the study question, clarify the hypothesis, determine an appropriate sample size and identify feasibility issues for prospective study. A retrospective study is one in which a search is made for a relationship between one (usually current) phenomenon or condition and another that occurred in the past (Mosby, 2008). A retrospective study could also be an epidemiological study in which participating individuals are classified as either having some outcome (cases) or lacking it (controls); the outcome may be a specific disease and the personal histories are examined for specific factors that might be associated with that outcome (O'Toole, 2003). The study period may be many years, but the time to complete the study is only as long as it takes to collate and analyze the data. Among the desirable attributes of a retrospective study is the ability to yield results from collectible data. It is adapted to the limited resources of an individual investigator and it places a premium on the formulation of hypotheses for testing rather than on facilities for data collection (Mantel and Haenszel, 1959).

Outbreaks of leptospirosis have occurred among general populations when people are exposed to flood waters that have high chances of leptospiral contamination (WHO 2000; Seghal *et al*., 2001).

Leptospirosis has now been recognized as a possible sequel of natural disasters such as cyclones and floods as during such times people and animals are exposed to wet environments for a prolonged period of time (Bal *et al*., 1994).

There have been no precise estimates of the global burden of human leptospirosis. At the first International Leptospirosis Society meeting in Nantes, France in 1996, a project to estimate the worldwide impact of human leptospirosis was initiated. The first report resulting from this effort was published by Smythe (1999). The goal was to collect reliable data on morbidity and mortality due to leptospirosis. The World Health Organization/Food and Agricultural Organization Collaborating Centre for Reference and Research on Leptospirosis in Queensland, Australia coordinated this project. Despite acknowledged flaws in the data with regard to completeness (passive reporting systems, unknown proportion of rural versus urban locations of disease, occupational and domestic risk factors, etc.) and perhaps diagnostic accuracy (diagnostic testing and criteria were not reported for any country), that report is of singular importance (Vinetz, 2001). This is because it is based on continuous global research that will provide the necessary disease burden data essential for the design of appropriate policy targeted towards decreasing the burden of Leptospirosis globally (WHO, 2010).

Leptospirosis is not limited to developing countries. Retrospective reviews of leptospirosis epidemiology have recently been reported from Ireland (Pate *et al*., 2000), Denmark (Holk *et al*., 2000) and Italy (Ciceroni *et al*., 2000). All of these reports point out the continued importance of leptospirosis as a public health issue that requires governmental efforts in surveillance and control (Vinetz, 2001). According to the OIE report (2005), the case fatality rate of leptospirosis in dog is approximately 10%.

This retrospective study was carried out to ascertain the occurrence of canine leptospirosis in Nigeria, to investigate the available mode of diagnosis and determine the morbidity and case fatality rates of the disease in Ibadan.

3.2 MATERIALS AND METHODS

3.2.1 Study area

The study area was Ibadan, Oyo State, Nigeria. Ibadan is located on latitude 7.3907^{o}N and longitude 3.8923^{o}E. The climate is equatorial with dry and wet seasons and relatively high humidity (NBS, 2012). It is located in southwestern Nigeria, 128km. inland northeast of Lagos and 530km. southwest of Abuja. Ibadan is a prominent transit point between the coastal region and areas to the North (http://en.wikipedia.org/wiki/Ibadan). The mean maximum temperature is 26.46 C, minimum 21.42 C and the relative humidity is 74.55%. The mean total rainfall for Ibadan is 1420.06 mm, falling in approximately 109 days (Fig. 3.1). There are two peaks for rainfall, June and September (BBC Weather, 2010).

3.2.2 Source of data

Two referral veterinary hospitals (a private veterinary clinic and a university veterinary teaching hospital) in Ibadan, southwestern Nigeria were used as case areas. These veterinary hospitals were purposively chosen for this study because of the following reasons:

They have high clientele base from within and outside Ibadan.

They keep clinical records.

The clinics cooperate with standard laboratories for post mortem examination of carcasses and accurate report.

The clinics have qualified veterinarians.

3.2.3 Method of diagnosis

Diagnosis of leptospirosis in these hospitals was based on history and clinical signs while confirmatory diagnosis was based on silver staining of leptospires in the kidney tissues at post mortem.

3.2.4 Study of hospital records

A retrospective study was done by going through the clinic records for the years 2005-2010. The occurrence of leptospirosis, morbidity and case fatality rates and the common clinical signs observed were noted.

Table 3.1 Climate data for Ibadan

Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Year
Record high °C (°F)	37 (99)	39 (102)	38 (100)	38 (100)	35 (95)	33 (91)	31 (88)	31 (88)	36 (97)	33 (91)	34 (93)	35 (95)	39 (102)
Average high °C (°F)	33 (91)	34 (93)	34 (93)	33 (91)	32 (90)	29 (84)	28 (82)	27 (81)	29 (84)	30 (86)	32 (90)	33 (91)	31 (88)
Average low °C (°F)	21 (70)	22 (72)	23 (73)	23 (73)	22 (72)	22 (72)	21 (70)	21 (70)	22 (72)	22 (72)	22 (72)	21 (70)	22 (72)
Record low °C (°F)	10 (50)	12 (54)	18 (64)	18 (64)	18 (64)	18 (64)	16 (61)	16 (61)	17 (63)	18 (64)	14 (57)	14 (57)	10 (50)
Rainfall mm (inches)	8 (0.31)	23 (0.91)	76 (2.99)	125 (4.92)	145 (5.71)	163 (6.42)	132 (5.2)	74 (2.91)	170 (6.69)	152 (5.98)	43 (1.69)	10 (0.39)	1,121 (44.13)
% humidity	76	71	75	78	82	86	88	88	86	84	80	76	81

Avg. precipitation days

Source: BBC Weather, 2010

The total number of cases for each month of the year for the entire period was calculated. The months were categorized into dry and rainy seasons. The months of November to March represented the dry season while the months of April to October represented the rainy season. The prevalence for each season was determined.

3.2.5 Data Analysis

The prevalence of leptospirosis for both dry and rainy seasons was compared to determine whether they differed significantly from each other. The Students' t-test was used for this.

3.3 RESULTS

3.3.1 Retrospective Study

A total of 5250 dog cases with different ailments presented at the private and public veterinary hospitals were reviewed for the period of 2005 to 2010. Out of these, 2463 (47.0%) were diagnosed as leptospirosis (Table 3.1). The case fatality rate was 37.0% (Table 3.2). Other cases treated at both veterinary hospitals within the stated period included babesiosis, helminthiasis, gastroenteritis, canine distemper, rabies, trauma / wound, ectoparasitism (mange, tick infestation), fungal infections, parvovirus infection, and poisoning. It was observed that most of the dogs that were taken to the clinic early enough after the onset of inappetence and diarrhea survived. Some of those that showed pain on kidney palpation also survived when the diagnosis and treatment were commenced early enough. They were usually treated with antibiotics, such as penicillin, penicillin/streptomycin combination or doxycycline. All the cases of mortality diagnosed as leptospirosis, some of which were confirmed by post mortem report from the Department of Veterinary Pathology of the University of Ibadan were those that were taken to the clinic at chronic stages, usually after being treated elsewhere for a wrong disease with clinical signs similar to leptospirosis. All such cases deteriorated to dyspnea in a position of lateral recumbency before death.

The monthly average number of cases diagnosed as leptospirosis average positive (205) was obviously less than the monthly average for the rainy season (245) and higher than the monthly average for the dry season (Table 3.4). The review showed a seasonal pattern of significantly more cases of leptospirosis occurring in the rainy season than in the dry season (Tables 3.5 and 3.6).

The results (Table 3.6) below show that there is a significant difference between the occurrence of leptospirosis during the rainy season and dry season (T-value= 7.49, degree of freedom = 4, $P=0.002 < 0.05$ level of Significance).

Thus, the general level of leptospirosis occurrence obtained during the rainy season was high (mean =3.036) and significantly different from the general level of occurrence obtained during the dry season (mean = 2.342).

Table 3.2 Total Leptospirosis morbidity rates for the study period (2005-2010).

Year	Total No. of Cases	Leptospirosis Morbidity	Morbidity rate %
2005	1093	471	43.0
2006	772	452	59.0
2007	1059	518	49.0
2008	863	345	40.0
2009	786	394	50.0
2010	677	283	42.0
Total	5250	2463	47.0

Table 3.3 Mortality and Case fatality rate for the study period (2005-2010).

Year	Total No. of Mortality	Mortality due to leptospirosis	Case fatality Rate (%)
2005	26	5	19
2006	16	12	75
2007	16	8	50
2008	17	3	18
2009	16	3	19
2010	17	9	53
Total	108	40	37.0

Table 3.4: General monthly occurrence of leptospirosis throughout the study period

Month	2005		2006		2007		2008		2009		2010	
	P	T	P	T	P	T	P	T	P	T	P	T
January	31	89	21	50	29	70	14	62	28	55	19	57
February	28	86	17	44	25	72	12	60	24	50	17	55
March	33	80	21	42	19	75	15	60	26	48	15	46
April	36	90	39	70	46	90	28	70	34	70	23	56
May	41	95	39	68	48	89	30	76	34	75	27	60
June	44	89	44	72	55	100	36	79	38	77	30	72
July	50	103	50	78	57	106	40	84	37	78	35	70
August	55	104	58	80	58	105	46	80	40	80	32	60
September	43	99	53	70	52	98	38	78	33	71	29	54
October	42	96	49	69	48	94	40	75	35	64	26	52
November	33	82	33	63	42	80	26	69	33	60	18	51
December	35	80	28	66	39	80	20	70	32	58	12	44
Total	471	1093	452	772	518	1059	345	863	394	786	283	677

Key: P - positive (Infected)

T -Total

Rainy season: April - October

Dry season: November - March

Total population of sample = 5250

Total positive = 2463

Table 3.5 Occurrence of leptospirosis cases during rainy and dry seasons

	Total No. of cases	Total diagnosed as leptospirosis	Average monthly cases diagnosed as leptospirosis
Rainy season April - October	3346	1718	245
Dry season November to March	1904	745	149
Total	5250	2463	205

Figure 3.1: Mean monthly prevalence of leptospirosis for the study period 2005 - 2010

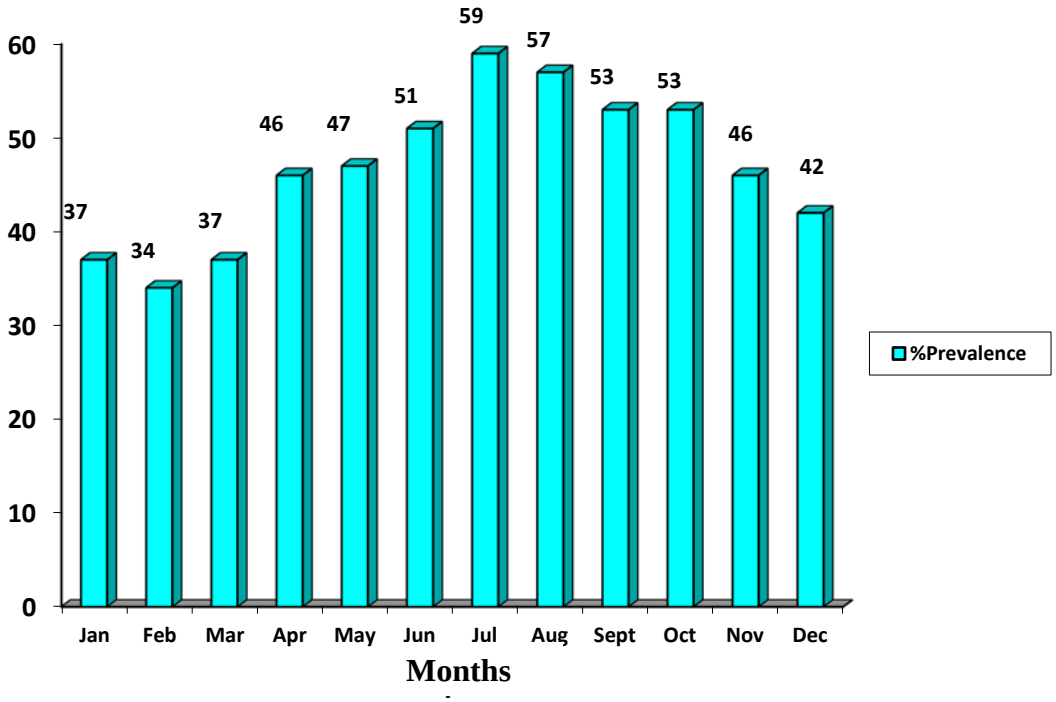


Table 3.6: Paired samples statistics showing the leptospirosis occurrence difference between the rainy and dry seasons of the study period 2005 -2010

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Rainy Season	3.0360	5	0.25677	0.11483
	Dry Season	2.3420	5	0 .24150	0.10800

Key N: Total number of samples

Table 3.7: **Paired samples test showing rainy and dry season occurrence of leptospirosis for the study period 2005 – 2010**

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Rainy - Dry Season	0.69400	0.20720	0.09266	0.43673	0.9512	7.490E0	4	0.002

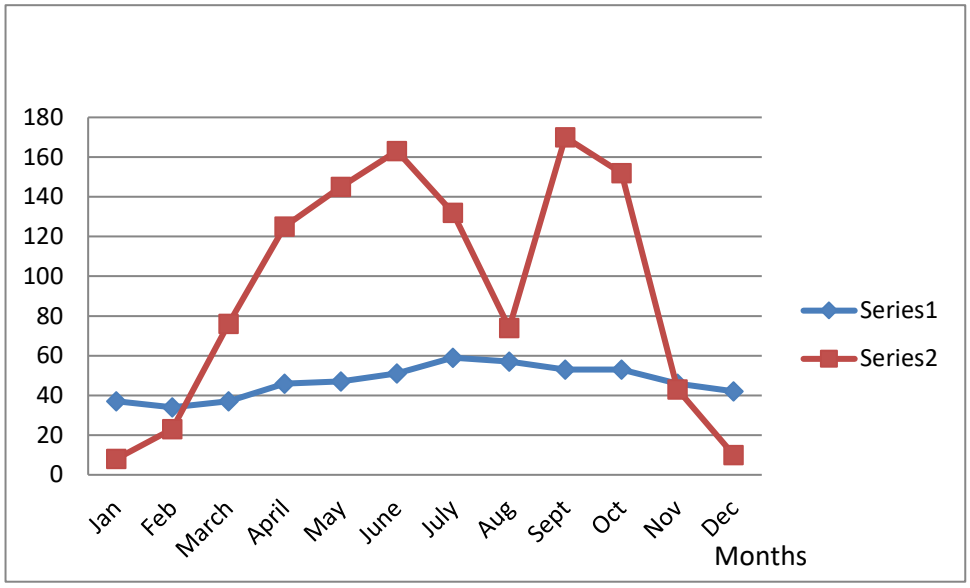
Key

t = statistical value

df= degree of freedom

Sig..= significance

Figure 3.2: Time-series of cases of canine leptospirosis reported at the veterinary hospitals and average monthly rainfall recorded at Ibadan (2005-2010)



Key

Series 1: Average monthly percentage prevalence of leptospirosis (%)

Series 2: Average monthly rainfall (mm)

3.4 DISCUSSION

The fact that leptospirosis was recorded as being responsible for 47.0% of all the clinical cases handled in the two referral veterinary hospitals in Ibadan for the six years reviewed, is an indication that leptospirosis is fast re-emerging. The figures obtained in this study show the consistent presence of leptospires in Nigeria. The case fatality rate (37.0%) obtained in this study is higher than the value 10-20% reported by Harkin and Gatrell (1996) and Birnbaum *et al.* (1998). This could be attributed to the fact that re-infection occurs when the environment is not thoroughly disinfected after previous infections and the prevailing environmental condition is favourable for the organism to thrive.

It was observed in this study that reports from both clinics showed that leptospirosis occurred all the year round and that it was significantly more prevalent in the rainy season than in the dry season. This is in agreement with the report of Faine (1994) that stated that the condition is more prevalent in the rainy season. Chen (1985), de Lima *et al.* (1990), Epstein *et al.* (1995), French and Holt (1989), Fuortes and Nettleman (1994), Oliveira *et al.* (1977), Pan American Health Organization (1998), Park *et al.* (1989), Simoes *et al.* (1969), Vanasco *et al.* (2000), World Health Organization (2000) all stated that cases of leptospirosis often follow a period of abundant flooding, usually during rainy season. The rainfall was at its peak in the month of June and this was followed by a peak in the prevalence of leptospirosis in the month of July. The drop in rainfall in the month of August could also be responsible for the slight drop in leptospirosis prevalence observed in the month of September, however, because the second peak in rainfall occurred in September, there was a plateau between September and October prevalence (Figure 3.2). According to Ward (2002), knowledge of the association between leptospirosis and rainfall allows pet owner education to be promoted to reduce exposure of dogs to leptospires. It also paves way to seasonally targeted vaccination.

Trevejo *et al*. (1998) had stated that dogs are important reservoir hosts for leptospires and a major source of environmental contamination. This may be due to the fact that dogs have a tendency to urinate indiscriminately in the environment. Urine from such dogs would greatly contaminate the environment. Lack of good drainage system accentuated

by frequent flooding experienced in Ibadan and other parts of Nigeria in recent times would create a multiplier effect on the role of infected dogs in the contamination of the environment and spread of leptospirosis. This is a cause for public health concern corroborated by Weekes *et al*. (1997) who stated dogs are a significant reservoir for human infection in many tropical countries and may be an important source of outbreaks.

CHAPTER FOUR

4.0 DETECTION OF LEPTOSPIRAL ORGANISMS BY THE USE OF DARK FIELD MICROSCOPE (CYTOVIVA™) WITH HYPERSPECTRAL IMAGING

4.1 INTRODUCTION

Dark field microscopy is a prompt diagnostic technique that is able to detect leptospires at a very early stage. Dark field microscopy of centrifuged urine is considered to be a convenient and rapid diagnostic test (Abdollahpour, 2011). Approximately 10^4 leptospires/ml are necessary for one cell per field to be visible by dark-field microscopy (Turner, 1970). In volunteers infected with serovar Grippotyphosa, leptospires were detected by DFM as early as 4 days prior to the development of symptoms (Alston and Broom, 1958). None of the DFM positive samples reported by Wolff (1954) were taken more than 6 days after onset of symptoms. Direct dark field microscopy of the cerebrospinal fluid of a patient without history of obvious exposure to leptospires taken 2 days after symptoms revealed many spirochaetes that were later confirmed by other tests to be pathogenic leptospires (Arzouni *et al*., 2002).

Optically-thin specimens are difficult to observe distinctly under normal light microscopes. Boustany *et al.* (2001) reported a variant of optical microscopy in the dark field termed optical scatter imaging. Another technique using hyperspectral imaging has been described by Lawrence (2006). The hyperspectral imaging technique is one that adds a colourful third dimension to a reflected image that contains the target's spectral data. Hyperspectral imaging microscopes deliver high spatial resolution and at the same time, high spectral resolution. Hyperspectral imaging combines digital imaging with spectroscopy (Durham, 2010). The author further stated that, unlike the human eye which sees only visible light, hyperspectral imaging can detect visible light as well as light from the ultra violet to near infra red ranges. The CytoViva™ dark field microscope with hyper spectral imaging system was an innovation of the Auburn University, United States of America introduced to the market in 2005. According to Foster (2004), conventional dark field takes images of an infinitely deep depth of field, including information from above

and below the plane of focus, creating haze and halo that obscure discrete particle information and distort image size. In comparison, CytoViva™ takes images of only a shallow plane, producing a crisp, clean background. Additionally, this shallow plane of focus offers the ability to perform optical sectioning, allowing the researcher to investigate cells layer by layer. While CytoViva™ images have a darkfield-like appearance, the optics go well beyond dark field to combine resolution with detection. The CytoViva™ system has been used to shed clearer light on the mechanism of spirochaete infestation (Foster, 2004). This CytoViva™ provides the following: An advanced high contrast optical microscope system, specifically designed for imaging nano-scale samples. A very near infra red (AVNIR) hyperspectral imaging capability that mounts unto the microscope system enabling spectral quantification of the sample being imaged.

The CytoViva™ microscope system is made up of the following components:

A high contrast illuminator which optimizes signal-to-noise ratio.

Dual Mode Fluorescence which enables labeled and unlabeled sample elements to be viewed independently or simultaneously.

Hyperspectral imager which quantifies sample elements through identification of their unique spectral signature.

Research grade imaging equipment made up of microscope, imaging camera, light source and environmental chamber

Annular illumination produces an improved point spread function. Through design, enhancement in the alignment and focus of annular illumination, CytoViva™ produces significantly improved optical performance over other comparable techniques including standard dark field (annular) illumination (Optics letters, 2006).

The following features contribute to the improved optical performance:

It has pre aligned Koehler illumination, which focuses the source light unto the entrance slit of the annular condenser.

It has a main feature of optical illumination which focuses the light precisely on the same plane of the sample as the objective focal point. This is made possible as a result of pre-aligned Koehler configuration.

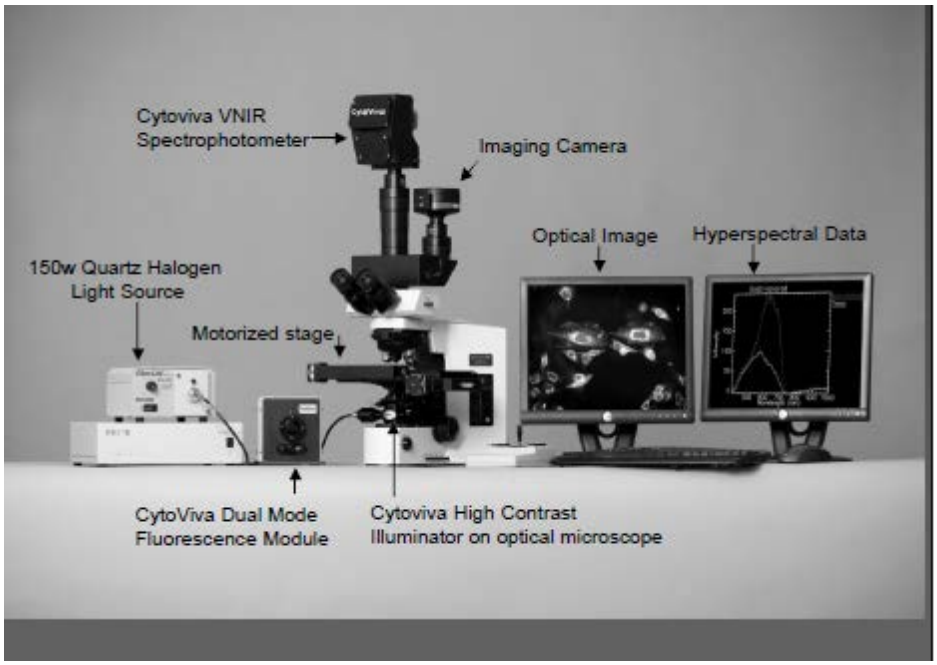


Plate 4.1 CytoViva™ Dark Field Microscope with Hyperspectral Imaging

Source: www.cytoviva.com

4.2: MATERIALS AND METHODS

4.2.1 Materials

Major Equipment:

Autoclave, water bath, pH meter, incubator, stirring machine with stirrer, refrigerator, inoculating hood, dark field CytoViva™ microscope with hyperspectral imaging system, stomacher, centrifuge.

Minor Equipment:

Conical flasks, 0.22µm millipore filter, 20 x 125mm sterile tubes with screw caps, micro-pipettes, micro tips (pipette tips), stomacher bags, glass slides, cover slips, bunsen burner, gloves, paper towels, markers, centrifuge tubes, Glass pipette (10ml), electric pipette pump.

Chemicals and Reagents

Furosemide (Lasix®) 0.5-1mg/kg, EMJH lepto media, distilled water, bacto agar, EMJH enrichment, phosphate buffered saline (PBS) and alcohol.

Test organisms and their source

The studied leptospires were obtained from the National Veterinary Services Laboratory (NVSL) US Department of Agriculture, Ames, Iowa. The species and serovars/strains used are shown in Table 4.1

4.2.2 Methods

4.2.2.1. Preparation of EMJH culture media

1.15g lepto media was carefully measured into the conical flask and 450ml distilled water was added to it. 1.00g Bacto agar was added to the mixture. The conical flask was placed on the stirring machine and a stirrer was put in it. When the content of the conical flask

was adequately mixed, it was removed from the stirring machine. The pH of the mixture was adjusted to 7.5±0.2 at 25ºC. The conical flask was placed on a hot plate and the content was allowed to boil. The conical flask was put in an autoclave cabinet, the temperature was set at 121ºC and allowed to run for 15 minutes. The conical flask was removed from the autoclave and placed in a water bath set at 56ºC to cool. The EMJH enrichment was removed from the refrigerator and placed on the work bench to attain room temperature after which it was filtered with a sterilized 0.22µm millipore filter. The filtered enrichment was aseptically added to the media and mixed thoroughly. 9ml of the media was carefully dispensed into each 20 x 125 mm sterile tube with screw cap. These were incubated overnight at 37ºC to check for sterility. They were stored at 29ºC.

4.2.2.2 Culture from pure isolates

Cultures were made from pure isolates of serovars Pomona, Hardjo, Autumnalis, Bratislava, Grippotyphosa, Icterohaemorrhagiae and Canicola into these semi-solid media in tubes and they were incubated at 29ºC. These tubes were inspected on a weekly basis for evidence of growth such as the dinger's ring. When such evidence was found, 10µl was aliquoted unto a clean slide, covered with a cover-slip and examined under the dark field (CytoViva™) microscope for the presence of leptospires.

4.2.2.3. Collection of Samples

Source of urine samples

The urine samples were collected from dairy farms A, B, and C located in Leesburg, Americus and Albany all in the south of Georgia State, U.S.A. The State of Georgia has a total area that spans 58,910sq. miles composed of 58,056sq. miles of land 158sq. miles of inland water. Georgia is bordered by Tennessee and North Carolina to its north, South Carolina to the east, Alabama to the west and Florida to the south. Georgia also borders the Atlantic ocean to the east (http://www.mapsofworld.com/usa/states/georgia). The study area for cattle urine collection has an annual average rainfall of 48.7 inhes and an average annual temperature of 29ºC from spring to fall (April – October) (http://www.usclimatedata.com/climate.php?lo).

Collection of urine samples

Cows on the farms visited had been routinely vaccinated against leptospirosis with vaccines containing serovars Hardjo and Pomona. On each farm thirty cows with history of infertility or repeat breeding were randomly selected for urine sample collection. The cows were given intramuscular injection of furosemide (Lasix®) at a dose of 0.5-1mg/kg to stimulate them to pass urine. The first micturition after administering the drug was ignored. Mid-stream urine of the second micturition after Lasix® administration was collected into labeled 40ml screw cap plastic containers. The farm name and the identification number on each cow were used as labels on the urine containers. These were taken to the laboratory for immediate processing and examination for leptospires.

Source of cattle kidney samples

The cattle kidneys were from a major beef harvest plant in Augusta, Georgia State, U.S.A. The beef harvest plant buys cattle from all over the United States. The cattle sent to the beef harvest plant were mainly males and some cows with history of reproductive failure. Sixteen kidneys from sixteen different cattle were randomly selected from the pool of kidneys at this plant. Each kidney was carefully placed in a new Ziploc® bag and all were placed in a plastic bowl and transported to the laboratory in an air-conditioned vehicle for immediate processing and examination.

Source of dog kidney samples

Kidneys were obtained from dogs that were euthanized at the dog shelter in Tifton. These dogs had no specific history as they were either stray dogs from unknown homes, old abandoned dogs or dogs sent to the shelter for re-housing. Tifton is in the south of Georgia State and has an annual average temperature of 29^{o}C from spring to fall, an annual average rainfall of 50 inches and hardly any record of snow (Table 5.1).

The kidneys were collected into new tightly sealed plastic containers that were labeled and taken immediately to the laboratory for processing and examination. An average of six dog kidneys were processed in a day as refrigeration or freezing of the tissues before processing was avoided.

Source of wildlife kidneys

Kidneys were harvested from wild animals that were killed at random by licensed hunters. These were relied on as sources of wildlife kidneys since it was unlawful for unlicensed individuals to kill wildlife in the State of Georgia. Kidneys were collected from a total of 31 wildlife composed of 5 bob cats, 2 beavers, 7 raccoons, 2 coyotes, 10 foxes and 2 opossums.

4.2.2.4. Processing and Dark field microscopy of Samples

Urine samples:

1.5ml of the urine sample was pipetted into each centrifuge tube and spun in the centrifuge at 14000 rpm at 25^{o}C for two minutes. A pipette was used to gently remove the supernatant leaving just a little bit above the pellet. The tube was refilled with about 1.4ml of urine and the procedure was repeated. After removing the supernatant, the pellet was rinsed by adding 1.5ml of phosphate buffered saline (PBS) to the pellet in the centrifuge tube. It was spun in the centrifuge at 14000rpm at 25^{o}C for two minutes. Most of the supernatant was removed with a pipette leaving just a little above the pellet. The pipette was used to mix the pellet with the small supernatant above it by aspirating and releasing the mixture in the tube a couple of times. From this sample, 15µl was aliquoted unto a clean slide, covered with a cover-slip a drop of the immersion oil was added and it was examined under the dark field microscope for the presence of leptospires.

Kidney samples:

A total of seventy seven kidney samples were used for this procedure. On each sample the following was carried out:

A piece of about 1g containing both the cortex and medulla was cut from the kidney sample. With a pair of forceps, it was dipped into a beaker of alcohol for a fraction of a second and quickly removed. Still held with the forceps, it was quickly passed through the bunsen burner flame to kill any microorganism on the surface. The sample was put in a stomacher bag, 8ml of PBS was added and it was placed in a stomacher which was run

for two minutes. The stomacher bag was removed from the stomacher and carefully placed on the bench for about ten minutes to allow the sample sediments to settle. A micropipette was used to add 15µl of the supernatant unto a clean slide, it was covered with a cover-slip and examined under the dark field microscope (CytoViva™) for the presence of leptospires using x100 oil immersion.

4.2.2.5 Determination of the rapidity of Dark field microscopy technique.

Urine samples.

The time required to carry out the procedure from the point of pipetting the first 1.5ml of urine into the centrifuge tube to the point of detecting a result under the microscope (rapidity) was recorded. A clock and a stop clock were set and used for this. The time of commencement of the procedure was recorded and the time it was completed was also recorded for each of the first ten samples and the mean value was used as the time taken to run a single test.

Kidney samples.

The time required to carry out the procedure from the point of cutting 1.0g piece of kidney to the point of obtaining result by observing the specimen under the fluorescent microscope was recorded. . A clock and a stop clock were set and used for this. The time of commencement of the procedure was recorded and the time it was completed was also recorded for each of the first ten samples and the mean value was used as the time taken to run a single test.

4.2.2.6 Determination of cost of DFM technique.

The fixed costs of the major equipment were calculated using their depreciation values. The variable costs which are the cost of consumable materials were calculated. This was used to calculate the operating cost per annum. (Appendices I and II).

Cost of total tests in a year		Y
Cost of tests in a month	(Y/12)	M
Cost of tests in a week	(M/4)	W
Cost of tests in a working day	(W/5)	D
Period of work in a day (9a.m.- 5p.m) 12noon-1p.m: lunch break	(420 min)	T
Time required to run through one test		T
Number of tests done daily	(T/t)	D
Cost per unit test	(D/d)	C

4.3 RESULTS

4.3.1. Results of dark field microscopy on culture of pure isolates and collected samples.

Pure isolates.

All the pure isolates cultured on the prepared semi-solid EMJH media were observable under the dark field microscope when examined a week after culture. However, the Borgpetersnii Hardjo organisms (*L. borgpetersenii* serovar Hardjo types A and B) designated Hardjo A and Hardjo B showed very minimal growth but they were still observable under the dark field (Cytoviva™) microscope (Table 4.1).

This proves that all the tested strains/serovars were sensitive to the dark field (cytoviva™) test. The test is not serovar-specific and neither can it differentiate between pathogenic and non pathogenic organisms.

Collected samples.

A total of 58 (34.73%) of 167 samples tested by dark field microscopy technique were positive for leptospirosis. Six out of 90 urine samples were positive.

The overall prevalence in kidneys was highest in the beavers (*Castor canadensis*) and coyotes (*Canis latrans*) with 100%, followed by dog *(Canis lupus familiaris*) with 83.3%, opossum (*Didelphis virginiana*) with 80% and cattle *(Bos Taurus*) with 75%. The details are shown in Table 4.2 and Figure 4.1. Some of the pictures are shown on plates 4.1 and 4.2.

Table 4.1: Pure isolates of leptospires cultured in semi-solid EMJH as examined by DFM

SEROVARS	DATES						
	30.06.08	07.07.07	14.07.08	21.07.08	28.07.08	04.08.08	11.08.08
Pomona	2+	3+	4+	3+	2+	3+	2+
Hardjo	1^{+}	1+	1+	2+	3+	4+	
Autumnalis	3+	2+	4+	4+	3+	2+	2+
Bratislava	4+	3+	3+	3+	3+	2+	1+
Grippotyphosa	2+	3+	4+	3+	3+	2+	2+
Icterohaemorrhagiae	2+	1+	4+	4+	3+	2+	2+
Canicola	2+	3+	4+	3+	3+	2+	2+
Tarassovi	1+	2+	3+	3+	2+	2+	2+
Biflexa	3+	3+	4+	4+	2+	2+	2+
Bjavanica	3+	2+	3+	2+	2+	2+	
Hardjo A	1+	1+	-	-	-	-	
Hardjo B	1+	1+	-	-	-	-	

Key
Negative –
Extremely Few 1+
Few 2+
Numerous 3+
Abundant 4+

Table 4.2 : Overall prevalence of leptospirosis as detected by DFM technique

Specimen	Species	No. tested	No. posistive	% positive
Urine	Cattle (*Bos taurus*)	90	6	6.7
Kidney	Cattle (*Bos taurus*)	16	12	75.0
Kidney	Dogs (*Canis lupus familiaris)*	30	25	83.3
Kidney	Bob cats (*Lynx rufus*)	5	2	40.0
Kidney	Beavers (*Castor canadensis*)	2	2	100
Kidney	Raccoon *(Procyon lotor*)	7	3	43.0
Kidney	Coyotes (*Canis latrans*)	2	2	100
Kidney	Foxes (*Vulpes vulpes*)	10	2	20.0
Kidney	Opossums(*Didelphis virginiana*)	5	4	80.0
	SUB-TOTAL KIDNEY	77	52	67.5
	GRAND TOTAL	167	58	34.7

Figure 4.1: Percentage of positive samples detected by Dark field microscopy

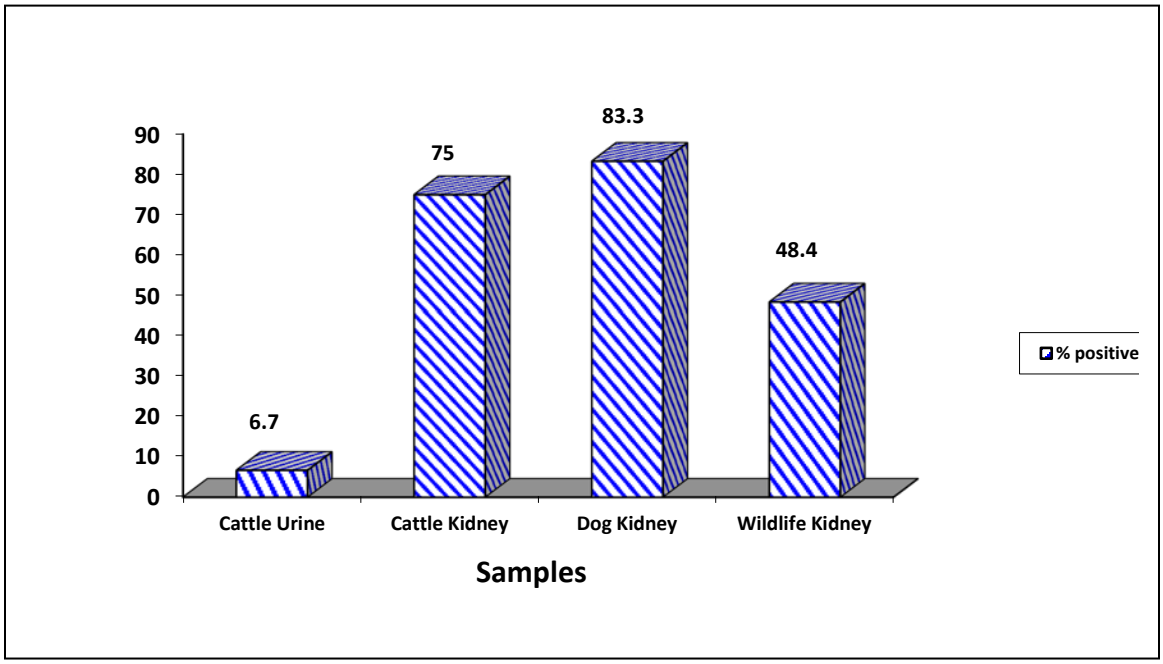


Plate 4.2 *Leptospira* displayed as viewed with the Cytoviva™ Dark Field Microscope. (Arrowed is the leptospire)

Magnification X100

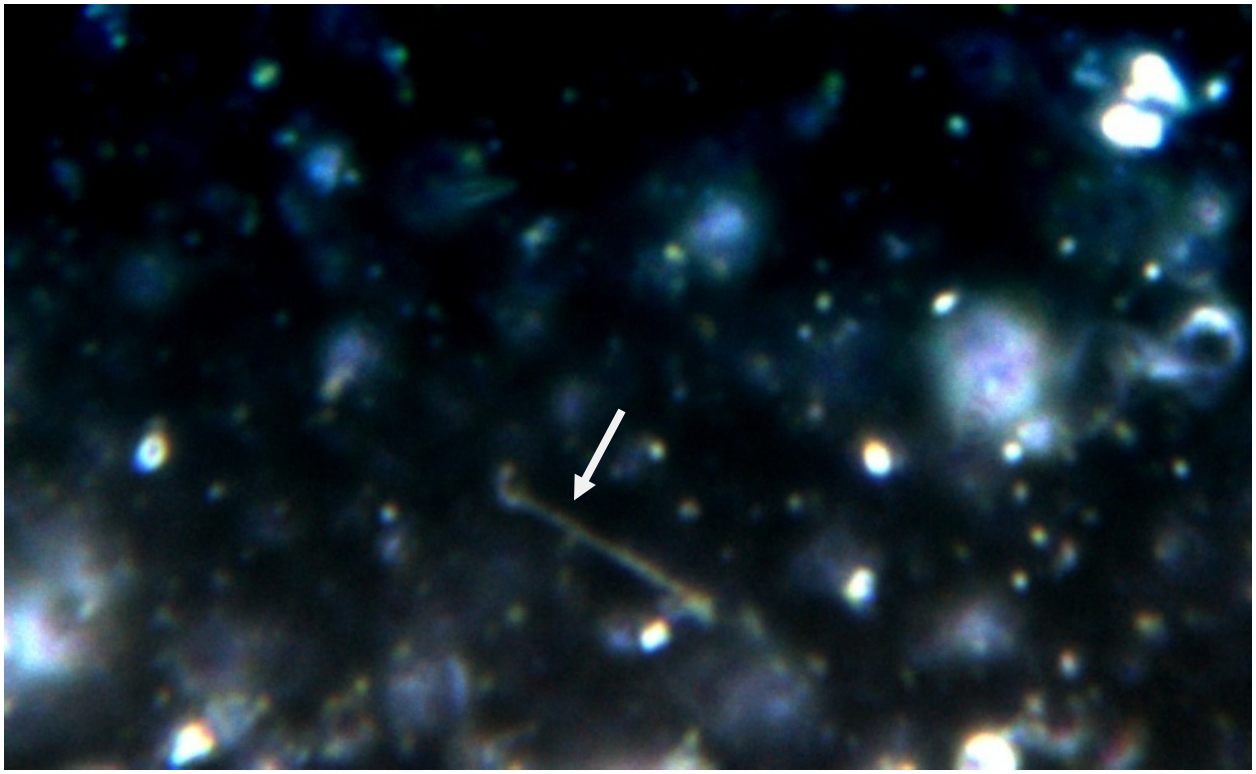
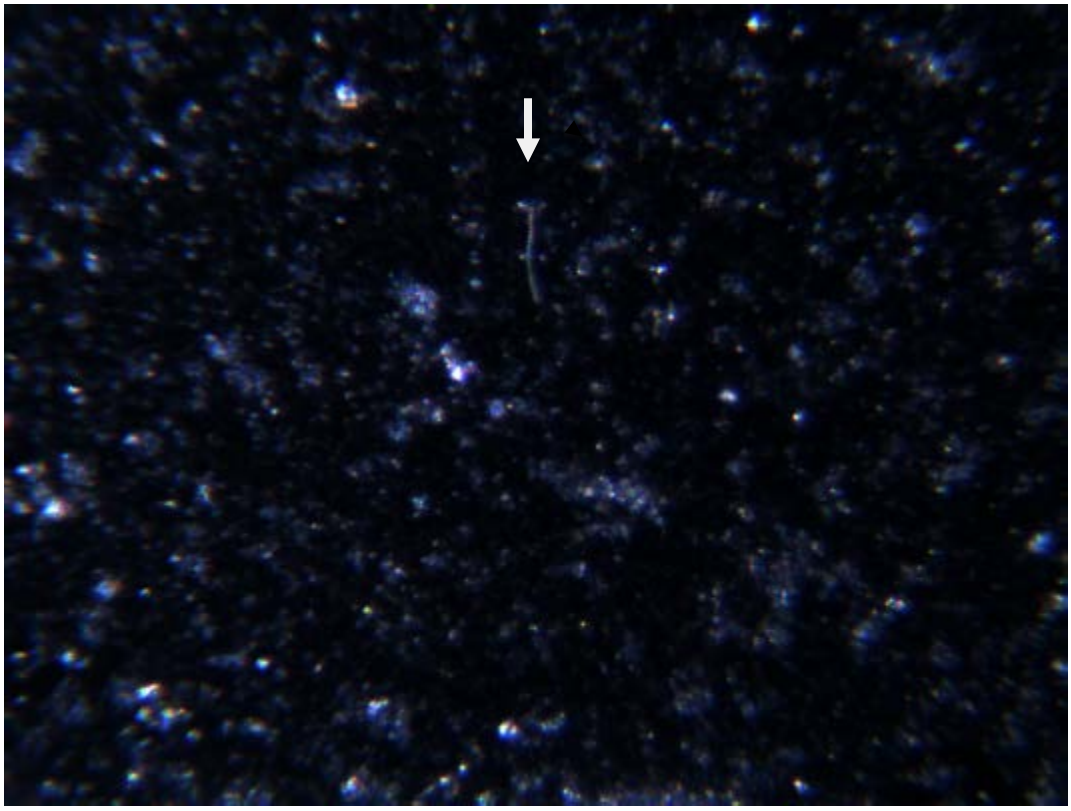


Plate 4.3 Dark Field Microscopy (CytoViva™) of *Leptospira* from cattle kidney sample. (Arrowed is the leptospire)

Magnification X100



4.3.2 Rapidity of the DFM technique

The time taken to conduct a DFM test was approximately 26.1 minutes.

4.3.3 Cost of the DFM technique per annum

*The cost of all the fixed equipment		₦596,960	
The cost of consumables (variable cost)		₦1,533,767	
**The cost of labour was		₦727,987.29	
The total cost was		₦2,858,714.29	
Cost per unit test			
Cost of total tests in a year		₦2,858,714.29	
Cost of tests in a month	(Y/12)	₦2,858,714.29/12	
Cost of tests in a week	(M/4)	₦238,226.2/4	(W)
Cost of tests in a working day	(W/5)	₦59,556.6/5	(D)
		₦11,911.32	
Period of work in a day		420min.	(T)
(9a.m.-5p.m; 1hr. lunch break)			
Time required to run a test		26.1min.	(t)
Number of tests done daily	(T/t)	16 tests	(d)
Cost per unit test	(D/d)	11,911.32/16	(C)
		₦744.6	

See Appendix I and II

Legend: Y= cost of total tests in a year

M= cost of total tests in a month

W= cost of total tests in a week

T= total amount of time in a working day

t= time required to carry out a single test

D= cost of tests in a working day

d = cost per unit test

*Depreciation value of fixed equipment was calculated as its unit cost divided by its lifespan.

**The cost of labour was taken as the salary per annum of a technologist (technologist I or senior technologist) to whom such a task and other similar assignments could be given.

4.4 DISCUSSION

A lot of previous work had been done on dark field microscopy of leptospires based on the use of the conventional dark field microscope. In this study, the recently developed CytoViva™ dark field microscope was able to detect the presence of all the pure isolates tested. The leptospires were distinctively observed and their hooked ends were very conspicuous. This is in agreement with (Chambers *et al.*, 1995; Foster, 2004) who stated that the CytoViva® dark field microscope has the ability to combine resolution with detection producing images in a crisp and clean background. The technique was sensitive to all the tested serovars. Turner (1970) had stated that leptospires must be present in a concentration of at least 10^4/ ml for them to be observed by dark field microscopy. The highest number of samples positive for leptospires was detected in the dog kidneys (25 positive samples) while the least was made in cattle urine (6 positive samples). This was probably due to the fact that the dairy cattle had been vaccinated against leptospirosis. The six positive samples detected despite vaccination were probably already infected before they were vaccinated and continued to shed the organism in their urine after vaccination. Such are refered to as 'chronic shedders' and should be culled. The positive samples could also be serovars that the administered vaccine did not protect against as discussed by Adler and Faine (1977) and Cole *et al*. (1982). The dogs were abandoned / stray dogs captured and kept in the shelter pending adoption into new homes or euthanization, so they had no evidence of previous vaccination. Detection of the organisms was also done in cattle kidney tissues from the beef harvest plant and from wildlife kidney tissues. It is pertinent to note that the DFM technique did not discriminate between pathogenic and non pathogenic leptospires. Hence some of the positive cases recorded might have been saprophytes. However, tests have been described by Johnson and Rogers (1964), Johnson and Harris (1967) and Johnson and Faine (1984) to distinguish between pathogenic and saprophytic leptospires. The ability of the CytoViva Dark Field Microscope to produce clearer images (Chambers *et al*., 1995) of leptospires with conspicuous hooks in addition to its ability to show a clearer view of the typical movement, make it less likely to confuse the organism with fibrils or artefacts.

The time taken to detect microorganisms from kidney tissues was longer than that taken for detection in urine samples using the DFM. Hence more samples may be examined per unit time using the DFM on urine samples than on kidney samples. The electric power required for the DFM technique was such as could be supplied by a portable generator in case of electric power outage.

CHAPTER FIVE

5.0 DETECTION OF LEPTOSPIRAL ORGANISMS BY FLUORESCENT ANTIBODY STAINING (FAS)

5.1 INTRODUCTION

Direct fluorescent antibody staining is another common method applied to veterinary specimens for diagnosis of *Leptospira* infection (Miller *et al*., 1989). Polyclonal antibody is required for this method. Out of seventeen (17) naturally infected dogs from which leptospires were eventually isolated, fourteen (14) were detected positive for leptospirosis by Fluorescent Antibody Staining of their urine samples (White *et al.,* 1961). Using FAS, some authors detected *L. canicola* in kidneys of hamsters and *L. pomona* in urine of guinea pigs and bovine and suggested that FAS was comparable to cultural methods when used on specimens containing relatively large numbers of leptospires (Boulanger and Robertson, 1961). Miller *et al.* (1989) reported some non specific fluorescence observed more in hamster kidney tissues than in bovine or porcine tissues. According to these authors, the short brightly fluorescing forms observed were believed to be leptospires with unusual morphology, described as short cells containing many inclusion bodies due to their particular physiological environment.

Fluorescent antibody staining technique is very useful for demonstrating leptospires in tissues from animals, (including fetuses that have died of leptospirosis (Cook *et al.,* 1972; Kirkbride and Halley, 1982). According to McDonough (2001), leptospires are commonly seen in the urine of seronegative carrier dogs and in dogs with clinical disease as early as 1 week post-infection with liver and kidney tissues being good specimens for FAS. Smith *et al.* (1966) reported that FAS was superior to culture and histopathological methods in demonstrating the presence of leptospires in autolysed materials. In fresh tissue homogenates containing live organisms, the supernatant fluid contained more organisms, whereas in autolyzed materials with dead organisms, the sediment was more likely to be positive. According to Hodges and Ekdahl (1973), FAS test of urine is a fast and accurate method for detecting the presence of leptospires. This method may be used in the discrimination of leptospires from artefacts (Bolin *et al*., 1989). Almost all

laboratories in the United States use fluorescent antibody staining for detecting leptospires at one stage or the other. When there are lots of other bacteria or faecal material present in the urine sample, it is difficult to find leptospiral organisms with fluorescent antibody staining (Wren, 2004). There is an understanding that when sampling a cattle herd for the presence of leptospires, fifteen animals are enough, regardless of the herd size. It was stated that fifteen animals would give a 95% chance of finding one test-positive animal, regardless of the herd size if the prevalence of infection is 20% or higher (Wren, 2004). Usually, when serovar Hardjo-bovis settles into an endemic situation, the infection rates are in the order of 20-30%. However, having fifteen negative tests on a big dairy does not mean there is no Hardjo-bovis present, but it does mean that the prevalence is unlikely to be 20% or higher (Wren, 2004).

5.2 MATERIALS AND METHOD

5.2.1 Materials

Major Equipment

Inoculating chamber with blower, fluorescent microscope, incubator, refrigerator, centrifuge, stomacher.

Minor Materials

Automatic pipettes, double-well glass slides for fluorescent microscopy, cover slips, micropipettes (50µl), pipette tips, slide rack, mounting oil, disposable inoculating loop, micro-tubes, paper towels, Petri dishes, bunsen burner, pairs of scissors, pairs of forceps, stomacher bags, beakers (100ml.) , sticks (tooth picks).

Biologicals and Chemicals

Rabbit anti-dog fluorescein isothiocyanate-conjugated antileptospira polyclonal antibody (FITC), pure isolates of *Leptospira*, urine samples, kidney samples, acetone, phosphate buffered saline, alcohol.

5.2.2 METHODS

5.2.2.1. Fluorescent antibody staining of organisms from pure isolate cultures

This served as the positive control.

Samples for this test were taken from the broth culture of pure isolates of *Leptospira* serovars described. For each serovar, 15µl of the sample was aliquoted within the wells on a double-well glass slide. It was gently spread with the use of a disposable inoculating loop and placed under a blower in an inoculating chamber to dry. On drying, each of the slides was placed in cold acetone for fifteen (15) minutes. The slides were removed from the acetone bath and gently placed on paper towels to dry. A drop of FITC-labelled *Leptospira* conjugate was added to each well of the glass slide. An inoculating loop was used to gently spread the drop to cover the whole well avoiding touching the slide with the loop. A humid environment was created in a large Petri dish by folding damp paper towels and placing them on two sticks (tooth picks) which had been carefully taped across the bottom of the dish to line it. The glass slides were placed on the damp towels and the petri dish was covered and placed in an incubator set at 37^{o}C for one hour. The Petri dish was removed from the incubator and each slide was gently rinsed by using a pipette to flush phosphate buffered saline (PBS) over it. Three beakers containing PBS were arranged in a row on the work bench. Each slide was immersed ten times into each of the beakers in quick succession. The slides were stood on paper towels to drain. The underside of each slide was dabbed with paper towels. The slides were gently shaken to remove any liquid retained on the upper side. 15µl of the mounting fluid was dropped within the well on each slide. It was covered with a cover slip and viewed under the fluorescent microscope at X40 magnification.

5.2.2.2 Source and Collection of samples.

Source of urine samples

The urine samples were collected from dairy farms A, B, and C located in Leesburg, Americus and Albany all in the south of Georgia State, U.S.A. The State of Georgia has a total area that spans 58,910sq. miles composed of 58,056sq. miles of land 158sq. miles of

inland water. Georgia is bordered by Tennessee and North Carolina to its north, South Carolina to the east, Alabama to the west and Florida to the south. Georgia also borders the Atlantic ocean to the east (http://www.mapsofworld.com/usa/states/georgia).

The study area for cattle urine collection has an annual average rainfall of 48.7 inhes and an average annual temperature of 29^{o}C from spring to fall (April – October) (http://www.usclimatedata.com/climate.php?lo).

Collection of urine samples

Cows on the farms visited had been routinely vaccinated against leptospirosis with vaccines containing serovars Hardjo and Pomona. On each farm thirty cows with history of infertility or repeat breeding were randomly selected for urine sample collection. The cows were given furosemide (Lasix®) at a dose of 0.5-1mg/kg intramuscularly to stimulate them to pass urine. The first micturition after administering the drug was ignored. Mid-stream urine of the second micturition after Lasix® administration was collected into labeled 40ml screw cap plastic containers. The farm name and the identification number on each cow were used as labels on the urine containers. These were taken to the laboratory for immediate processing and examination for leptospires.

Source of cattle kidney samples

The cattle kidneys were from a major beef harvest plant in Augusta, Georgia State, U.S.A. The beef harvest plant buys cattle from all over the United States. The cattle sent to the beef harvest plant were mainly males and some cows with history of reproductive failure. Sixteen kidneys from sixteen different cattle were randomly selected from the pool of kidneys at this plant. Each kidney was carefully placed in a new Ziploc® bag and all were placed in a plastic bowl and transported to the laboratory in an air-conditioned vehicle for immediate processing and examination.

Source of dog kidney samples

Kidneys were obtained from dogs that were euthanized at the dog shelter in Tifton. These dogs had no specific history as they were either stray dogs from unknown homes, old abandoned dogs or dogs sent to the shelter for re-housing. Tifton is in the south of

Georgia State and has an annual average temperature of 29^{o}C from spring to fall, an annual average rainfall of 50 inches and hardly any record of snow (Table 4.3).

Each pair of kidneys was collected into new tightly sealed plastic containers that were labeled and taken immediately to the laboratory for processing and examination. An average of six dog kidneys were processed in a day as refrigeration or freezing of the tissues before processing was avoided.

Source of wildlife kidneys

Kidneys were harvested from wild animals that were killed at random by licensed hunters. These were relied on as sources of wildlife kidneys since it was unlawful for unlicensed individuals to kill wildlife in the State of Georgia. Kidneys were collected from a total of 31 wildlife composed of 5 bob cats, 2 beavers, 7 raccoons, 2 coyotes, 10 foxes and 2 opossums.

Figure 5.1 Map of Georgia State, U.S.A.

Source: www.mapsofworld.com

Table 5.1: Climate - Tifton - Georgia

Temperature - Precipitation ^{o}C

	Jan	Feb	March	April	May	June
Average high in °C	15	17	21	24	28	31
Average low in °C	3	5	8	12	16	20
Av. precipitation - mm	135	110	128	88	81	104

	July	Aug	Sep	Oct	Nov	Dec
Average high in °C	32	32	30	26	21	16
Average low in °C	22	21	19	13	8	4
Av. precipitation - mm	115	104	88	66	81	93

Source: http://www.usclimatedata.com/climate.php?location=USGA0568

Table 5.2: Climate - Americus - Georgia

Temperature - Precipitation ^{o}C

	Jan	Feb	March	April	May	June
Average high in °C	14	17	21	25	28	32
Average low in °C	1	2	6	9	14	18
Av. precipitation - mm	140	125	131	94	89	100

	July	Aug	Sep	Oct	Nov	Dec
Average high in °C	33	32	30	25	20	15
Average low in °C	20	20	17	11	6	3
Av. precipitation - mm	136	90	85	58	94	105

Source: http://www.usclimatedata.com/climate.php?location=USGA0017

5.2.2.3 Processing and Fluorescent Antibody Staining of Samples

Urine samples:

1.5ml of the urine sample was pipetted into each centrifuge tube and spun in the centrifuge at 14000 rpm at 25^{o}C for two minutes. A pipette was used to gently remove the supernatant leaving just a little bit above the pellet. The tube was refilled with about 1.4ml of urine and the procedure was repeated. After removing the supernatant, the pellet was rinsed by adding 1.5ml of phosphate buffered saline (PBS) to the pellet in the centrifuge tube. It was spun in the centrifuge at 14000rpm at 25^{o}C for two minutes. Most of the supernatant was removed with a pipette leaving just a little above the pellet. The pipette was used to mix the pellet with the small supernatant above it by aspirating and releasing the mixture in the tube a couple of times. From this pellet, 15µl was aliquoted within the wells on a double-well glass slide. It was gently spread with the use of a disposable inoculating loop and placed under a blower in an inoculating chamber to dry. On drying, each of the slides was placed in cold acetone for fifteen (15) minutes. The slides were removed from the acetone bath and gently placed on paper towels to dry. A drop of FITC-labelled *Leptospira* conjugate was added to each well of the glass slide. An inoculating loop was used to gently spread the drop to cover the whole well avoiding touching the slide with the loop. A humid environment was created in a large Petri dish by folding damp paper towels and placing them on two sticks (tooth picks) which had been carefully taped across the bottom of the dish to line it. The glass slides were placed on the damp towels and the Petri dish was covered and placed in an incubator set at 37^{o}C for one hour. The Petri dish was removed from the incubator and each slide was gently rinsed by using a pipette to flush phosphate buffered saline (PBS) over it. Three beakers containing PBS were arranged in a row on the work bench. Each slide was immersed ten times into each of the beakers in quick succession. The slides were stood on paper towels to drain. The underside of each slide was dabbed with paper towels. The slides were gently shaken to remove any liquid retained on the upper side. 15µl of the mounting fluid was dropped within the well on each slide. It was covered with a cover slip and viewed under the fluorescent microscope at X40 magnification.

5.2.2.4. Determination of the rapidity of Fluorescent Antibody Staining technique

Urine samples.

The time required to carry out the procedure from the point of pipetting the first 1.5ml of urine into the centrifuge tube to the point of detecting a result under the fluorescent microscope (rapidity) was recorded. A clock and a stop clock were set and used for this. The time of commencement of the procedure was recorded and the time it was completed was also recorded for each of the first ten samples and the mean value was used as the time taken to run a single test.

Kidney samples.

The time required to carry out the procedure from the point of cutting 1.0g piece of kidney to the point of obtaining result by observing the specimen under the fluorescent microscope was recorded. A clock and a stop clock were set and used for this. The time of commencement of the procedure was recorded and the time it was completed was also recorded for each of the first ten samples and the mean value was used as the time taken to run a single test.

5.2.2.5. Determination of the cost of Fluorescent antibody staining technique

The fixed costs of the major equipment were calculated using their depreciation values. This was done by dividing the unit cost of the equipment by the lifespan of the equipment. The variable costs which are the cost of consumable materials were calculated.

This was used to calculate the operating cost per annum. (Appendix III and IV)

Cost of total tests in a year		Y
Cost of tests in a month	(Y/12)	M
Cost of tests in a week	(M/4)	W
Cost of tests in a working day	(W/5)	D
Period of work in a day (9a.m.- 5p.m) 12noon to 1p.m. lunch break	(420 min)	T
Time required to run through one test		T
Number of tests done daily	(T/t)	D
Cost per unit test	(D/d)	C

5.3 RESULTS

5.3.1 Fluorescent antibody staining of Pure Isolate Cultures and Samples

All the slides prepared with pure isolates of serovars (Pomona, Hardjo, Autumnalis, Bratislava, Grippotyphosa, Icterohaemorrhagiae, Canicola, Tarassovi, Biflexa, Bjavanica, Hardjo type A and Hardjo type B) obtained from NVSL, US Department of Agriculture, Ames, Iowa showed clear positive results. The indication of a positive result is a slide with typical morphology of *Leptospira* and positive fluorescence (Miller *et al*., 1991). This was used as control test before the field samples were tested with the same procedure. Fluorescent antibody staining of cattle urine samples from dairy showed that six (6.7%) of the ninety samples were positive (one of the positive urine samples is shown in Plate 5.1) and 30 (39%) of all the kidney samples were positive. A total of 36 (21.55%) of the urine and kidney samples were positive. Highest prevalence of 18 (60%) were observed in dog kidney samples, followed by cattle kidney with 56% and opossum with 40% (Table 5.1 and Figure 5.1).

Among the 31 wildlife samples 18 (58.1%) were recorded as suspects because the stained organisms appeared fragmented as shown in Plate 5.4. Examples of positive samples are shown in Plate 5.2 for opossum and Plate 5.3 for dog.

Table 5.3: Overall prevalence of leptospirosis as detected by FAS technique

Specimen	Species	No. tested	No. posistive	% positive
Urine	Cattle (*Bos taurus*)	90	6	6.7
Kidney	Cattle (*Bos taurus*)	16	9	56.0
Kidney	Dogs (*Canis lupus familiaris)*	30	18	60.0
Kidney	Bob cats (*Lynx rufus*)	5	0	0
Kidney	Beavers (*Castor canadensis*)	2	0	0
Kidney	Raccoon *(Procyon lotor*)	7	0	0
Kidney	Coyotes (*Canis latrans*)	2	0	0
Kidney	Foxes (*Vulpes vulpes*)	10	1	10.0
Kidney	Opossums(*Didelphis Virginiana*)	5	2	40.0
	SUB-TOTAL KIDNEY	77	30	39.0
	GRAND TOTAL	167	36	21.6

Figure 5.2: Percentage of positive samples detected by Fluorescent Antibody Staining

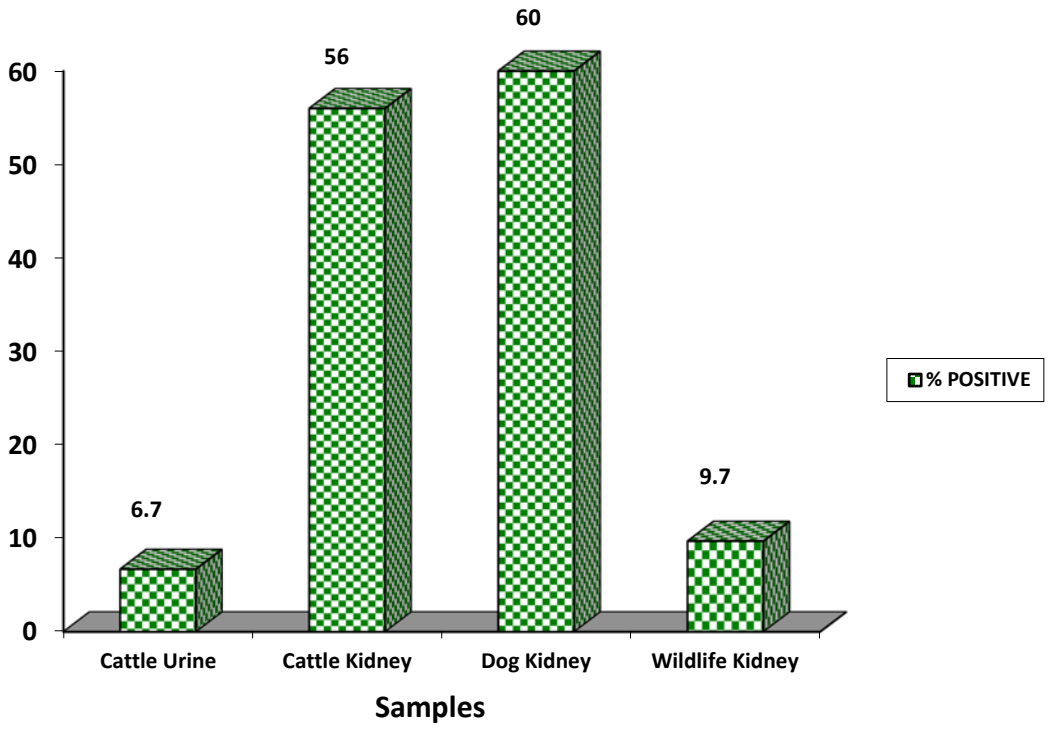
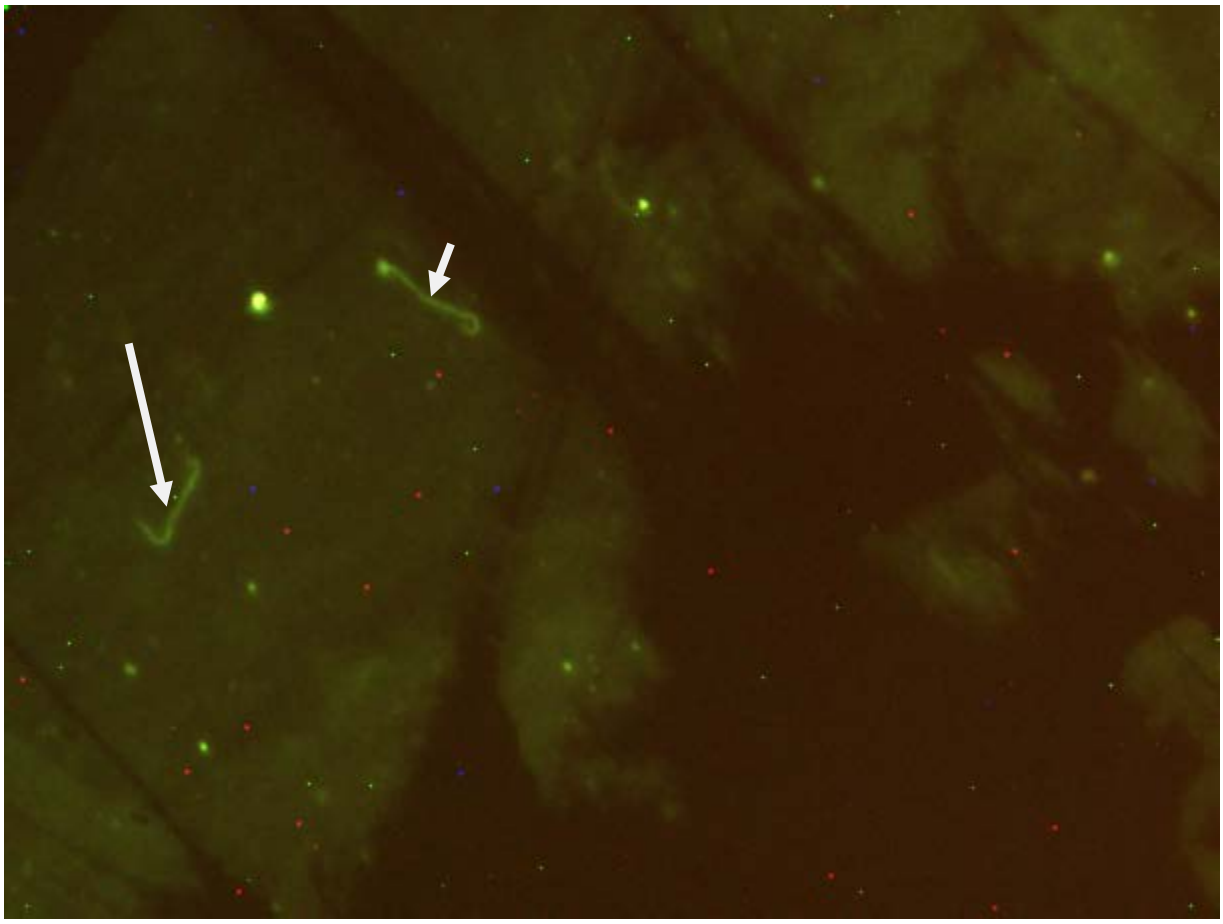
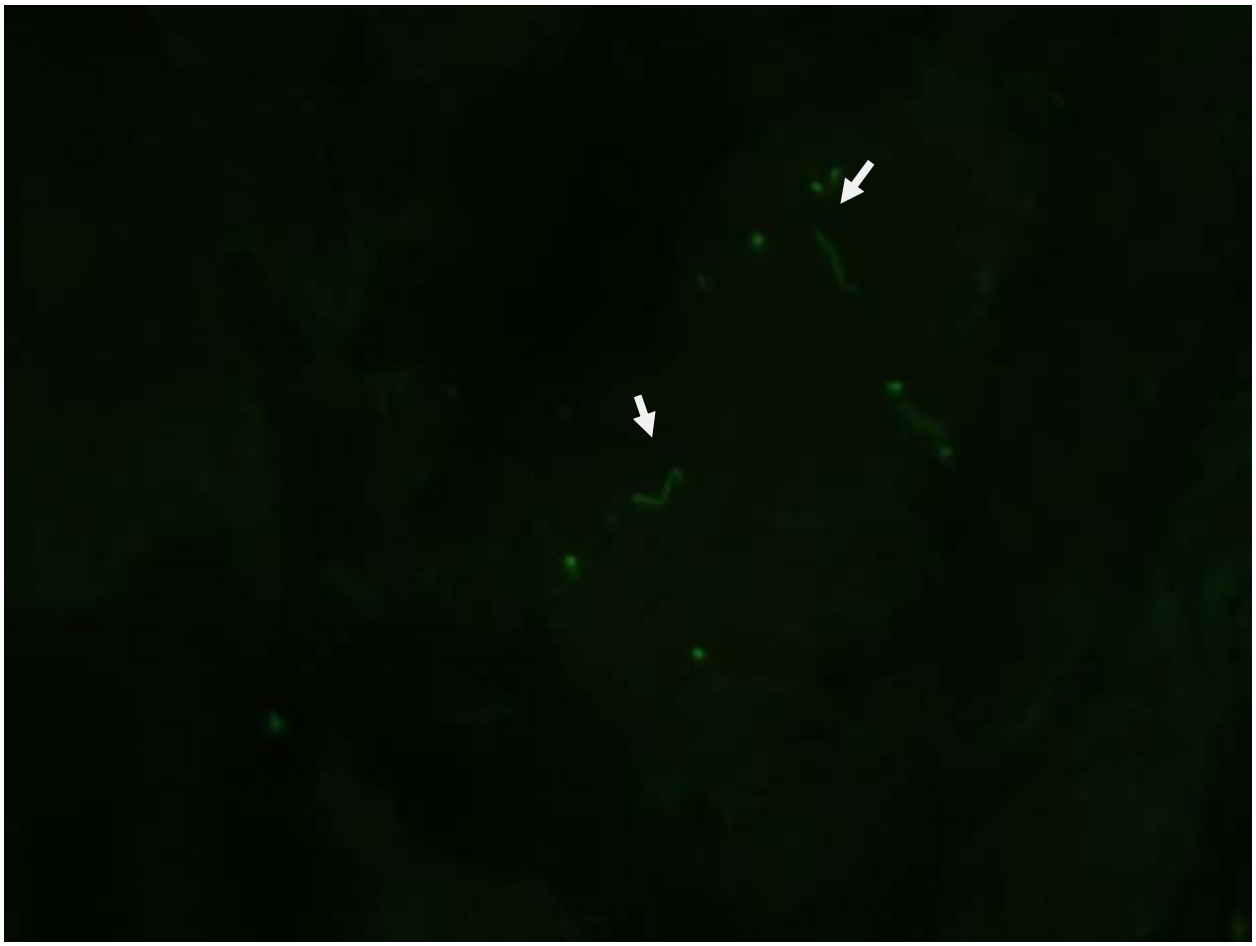


Plate 5.1: Fluorescent antibody staining of cattle urine sample from dairy cattle



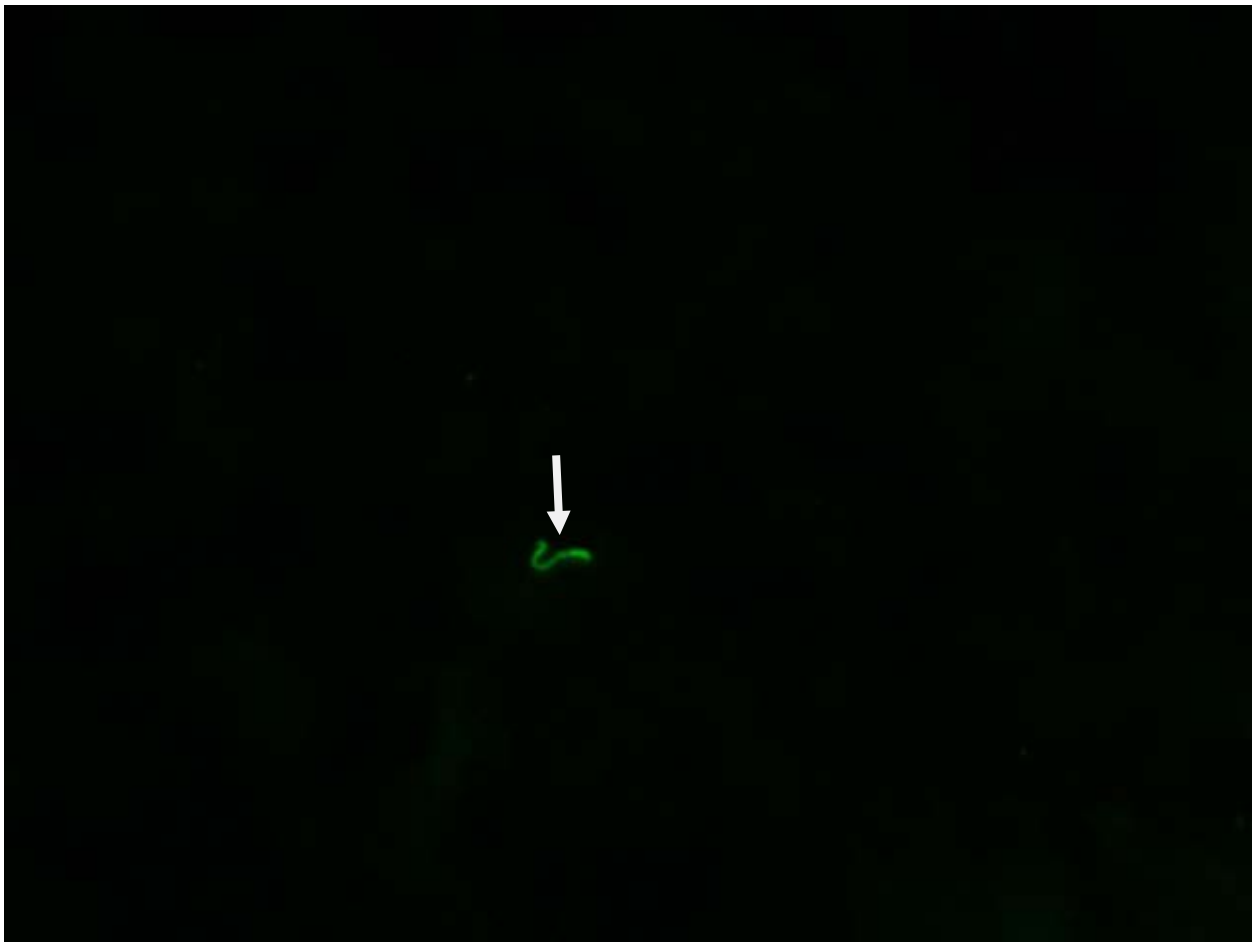
Key: (Arrowed are the leptospires).

Plate 5.2: Fluorescent Antibody Staining of opossum kidney tissue sample



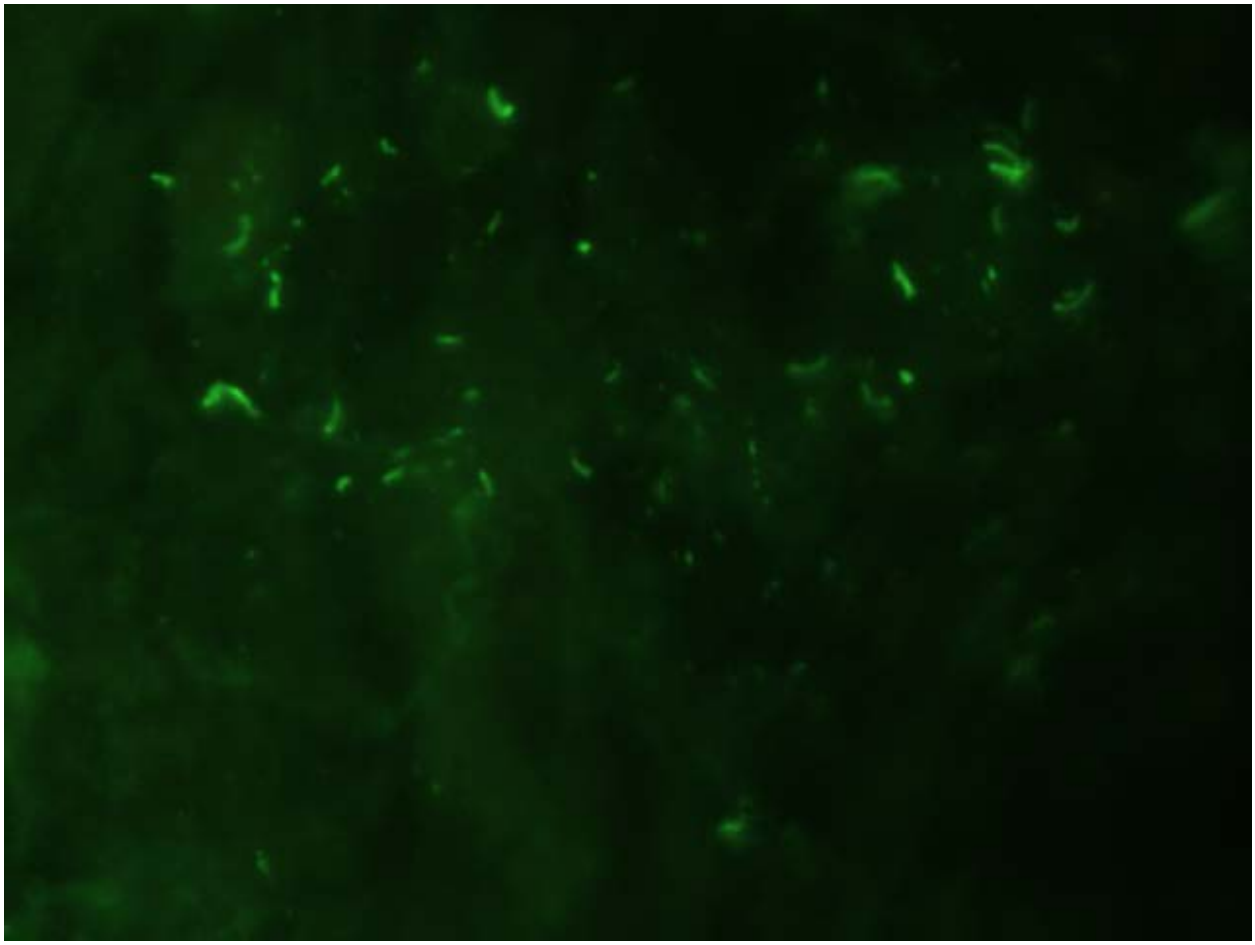
Key: Arrowed are the leptospires

Plate 5.3: Fluorescent antibody staining of Dog kidney tissue sample



(Arrowed is the leptospire)

Plate 5.4: Fluorescent antibody staining of wildlife kidney tissue sample (referred to as 'suspect')



5.3.2: Rapidity of FAS technique.

The results showed that the average time required to run a test using the FAS was 120 minutes.

5.3.3: Cost of operation of FAS technique per annum.

Cost of fixed equipment/materials	₦275,960
Cost of consumables (variable cost)	₦719,732.5
Cost of labour	₦900,000
Total cost per annum	₦1,895,692.5

Cost per unit test

Cost of total tests in a year		₦1,895,692.5
Cost of tests in a month	(Y/12)	₦1,895,692.5/12
		₦157,974.4
Cost of tests in a week	(M/4)	W =157,974.4/4
		₦39493.6
Cost of tests in a working day	(W/5)	D = ₦39493.6/5
		₦7898.72
Period of work in a day (9a.m.- 5p.m)		T = 420 min.
Time required to run through one test		t = 120 min.

Number of tests done daily	(T/t)	d = 4tests
Cost per unit test	(D/d)	C = ₦ 7898.72/4
		₦1974.68

See Appendices III and IV

Legend: Y= cost of total tests in a year t= time required to carry out a test

M= cost of total tests in a month

W= cost of total tests in a week D= cost of tests in a day

T= total amount of time in a working day d= number of tests per day

5.4 DISCUSSION

The reason for the high percentage (60%) of dogs tested positive by FAS for leptospires is mainly because the dogs were stray or abandoned dogs. They are reservoir hosts to some serovars such as Canicola. The dogs had little chance of being treated with any antibiotics which might have cleared out the organisms. They were probably not vaccinated against the circulating serovars to which they were exposed. The prevailing climatic condition in Tifton from April to October (Table 5.1) favours the survival of the organisms in the environment, especially in the soil and surface waters.

The kidney samples from the beef harvest plant when tested by FAS showed 56% as positive. This high value could be due to the fact that the cattle that were either infertile or showed “repeat breeding” were often culled by sending them to the beef harvest plant. From the results obtained in this work, it could also be concluded that leptospirosis might be one of the causes of infertility, as Bolin (2003) stated that leptospirosis could be responsible for infertility in cattle.

The least positive results for FAS (6.7%) was obtained from the cattle urine samples. This was probably due to the fact that these cattle had been routinely vaccinated against leptospirosis. The remarkable morphology of the organism stained fluorescent was captured in Fig. 5.1 with the conspicuous hooked ends as described by Miller *et al.* (1991). The seemingly low percentage of positive samples obtained despite vaccination was probably due to infection by serovars not covered by the vaccines administered because vaccines are serovar specific (Adler and Faine, 1977; Cole *et al*., 1982; and Rakesh *et al*., 1989). However, Wren (2004) reported that fifteen animals would give a 95% chance of finding one test-positive animal, in any herd, regardless of the herd size if the prevalence of infection is 20% or higher. Since six cows were positive among ninety there is a possibility that one could have been found positive in every group of fifteen cows tested. According to the author, when serovar Hardjo-bovis settles into an endemic situation, the infection rates are usually on the order of 20-30%. It could therefore be further concluded that even though only six samples were positive out of a herd of ninety, the prevalence of infection was 20% or higher in the entire dairy herd. In order to prevent

a state of endemic leptospirosis on these farms the animals that were positive for leptospirosis should be culled. Further tests should be done to isolate the serovars present and efforts should be made to use vaccines that are effective against the serovars present.

The observed percentage of positive samples detected by FAS in the wildlife samples was relatively lower than what was obtained by DFM. However, the percentage of samples that were termed “suspects” (58.06%) was relatively high. These appeared fragmented, even though they took up the fluorescent stain (Plate 5.4). Similar observation was made in hamster kidneys by Miller *et al*., (1989). These authors claimed that the unusual morphology was due to the particular physiological environment. Further work should be done to ascertain whether such should be classified as outright positive or negative.

The rapidity test showed that an average time of 120 minutes was required to run the FAS. The running of the FAS required skill in order to ensure that none of the steps was skipped. The operating cost per unit test for FAS was ₦1974.68. The evaluation showed that it is a prompt and relatively affordable test.

The FAS was observed to be a reliable and sensitive test and the results were easy to interprete. However, it is not serovar specific and cannot distinguish between pathogenic and non pathogenic leptospires. This is in agreement with the reports of Miller *et al.* (1991) and Bolin (2003).

CHAPTER SIX

6.0 DETECTION OF LEPTOSPIRAL ORGANISMS BY CONVENTIONAL POLYMERASE CHAIN REACTION (PCR)

6.1 INTRODUCTION

The polymerase chain reaction (PCR) is a technique that amplifies a DNA template to produce specific DNA fragments in vitro. It is used to amplify DNA fragments and detect DNA or RNA sequences within a cell or environment. Each cycle of PCR includes steps for template denaturation, primer annealing and primer extension. The initial step denatures the target DNA by heating it to 94°C or higher for 15 seconds to 2 minutes. In the denaturation process, the two intertwined strands of DNA separate from one another, producing the necessary single-stranded DNA template for replication by the thermostable DNA polymerase. In the next step of a cycle, the temperature is reduced to approximately 40–60°C. At this temperature, the oligonucleotide primers can form stable associations (anneal) with the denatured target DNA and serve as primers for the DNA polymerase. This step lasts approximately 15–60 seconds. Finally, the synthesis of new DNA begins as the reaction temperature is raised to the optimum for the DNA polymerase. For most thermostable DNA polymerases, this temperature is in the range of 70–74°C. The extension step lasts approximately 1–2 minutes. The next cycle begins with a return to 94°C for denaturation. In leptospirosis study, PCR involves the amplification of specific fragments of leptospiral genomic DNA in clinical samples. This reaction requires the selection of specific primers to allow amplification of all strains that are classified as pathogenic or potentially pathogenic (Faine *et al.,* 1999). According to Merien *et al*. (1992), the two main targets have been a unique set of primers derived from leptospiral ribosomal 16S rRNA gene (rrs gene) demonstrated as universal for the species of Leptospira or a combination of two sets of primers derived from genomic libraries of serovars icterohaemorrhagiae and bim. G1/G2 are primers which amplify the target DNA from all the serovars including non-pathogenic Leptospira biflexa whereas LP1/LP2 and Lig1/Lig2 primers amplify only pathogenic leptospires (Palaniappan *et al.,* 2005). Molecular tools such as conventional and real time PCR are considered as

sensitive and specific assays for the rapid detection of leptospires during early stages of infection (Gravekampe *et al.,* 1993; Merien *et al.,* 1993). Conventional PCR assay with G1/G2 primers has been shown to be specific for leptospires including non pathogenic leptospires. The persistence of non-pathogenic Leptospira biflexa in the filter sterilized water (Rubin *et al.,* 1980), laboratory (Koizumi *et al.,* 2003) and also in the normal equine kidney (Myers, 1976) may confuse the diagnosticians, ultimately leading to false positive results. Therefore, developing primers that can differentiate non-pathogenic from pathogenic serovars during PCR assay has proven to be a challenge for molecular diagnosticians. Test sensitivity depends on the ability of the primers to detect pathogenic species of the genus circulating in the population plus the type of PCR adopted by the laboratory (Smythe *et al*., 2002).

The validity of PCR data depends essentially on the quality controls included in the test. Although PCR technology is now widely used for the diagnosis of many diseases, its general value for the rapid diagnosis of leptospirosis has not been evaluated worldwide as it is not yet widely used, particularly in tropical and subtropical countries (WHO, 2003). Although previous reports indicated that PCR was promising for the early diagnosis of leptospirosis (Levett, 2001]), it still has not found widespread use outside of research or reference laboratories (Vinetz, 2001).

6.2 MATERIALS AND METHODS

6.2.1 Materials

Major Equipment: Thermal cycler (Gradient™), refrigerator, freezers (-20^{o}C, -80^{O}C), microwave oven, Mettler weighing balance, centrifuge machine, mini centrifuge machine, vortex shaker, gel documentation and analysis system (with U-V illuminator and Kodak Polaroid camera), DNA work station, gel trays.

Minor Equipment: micropipettes, microtips, centrifuge tubes, microtubes, racks for microtubes, PCR tubes, racks for PCR tubes, stop clock, gloves, paper towels

Biologicals and Reagents*:* Ethidium bromide, Mo Bio™ Ultraclean DNA kit, PBS, Extracted DNA of pure *Leptospira* isolates, master mix, 100bp ladder, Tris-Acetate (TAE), nuclease-free water, primers.

6.2.2 Methods

6.2.2.1 Evaluation of Polymerase Chain Reaction using multiple primer sets for the detection of Leptospira serovars.

A trial test was carried out using PCR on pure isolates of 12 serovars of leptospires.

Ten primer sets (Table 6.1) were used for detecting twelve serovars of *Leptospira*, belonging to three species. The serovars included the seven *L. interrogans* (LI), four *L. borgpetersenii* (LB) and one saprophytic species *L. biflexa* (LBF).

Table 6.1 List of Primer pairs, base product sizes and sequences

S/N	PRIMERS	SIZES	GENE SEQUENCES
1	LIP32	423bp	5' CGCTGAAATGGGAGTTCG TATGATT-3'
			5'CCAACAGATGCAACGAAAG ATCCTTT-3'
2	GYR_1	502bp	
3	G_1	285bp	5'CTGAATCGCTGTATAAAAGT
	G_2		5'GGAAAACAAATGGTCGGAAG
4	LP1	274bp	5'ATACAACTTAGGAAGAGCAT-3'
	LP2		5'GCTTCTTTGATATAGATCAA-3'
5	P_1	650bp	5'TTCGATTCAAAGCATGGCTAACG-3'
	M_{16}		5'AAAGAAGGACTCAGCGACTGCG-3'
6	LEP1	493bp	5' GTCAAACGGGTAGCATACC3'
	2R		5' GTCCGCCTACACACCCTTTAC3'
7	$L16R_F$	330bp	5'GGCGGCGCGTCTTAAACATG3'
			3'AATCTTGCTCAATGGGGGGAA5'
8	737	482bp	+GCAAGCATTACCGCTTGTGG
	1218		-TGTTGGGGAAATCATACGAAC
9	L_1R_1	520bp	
10	$B_{64\,I}$	563bp	5'ACTAACTGAGAAACTTCTAC
	$B64\,II$		5'TCCTTAAGTCGAACCTATGA

Table 6.2: Showing the PCR results of 14 primer sets against 12 leptospiral serovars

Species	Serovar/ strain	PCR Results for Primer sets									
		1	2	3	4	5	6	7	8	9	10
LI	Autumnalis, Akiyami A	P	P	P	P	P	P	P	N	N	P
LI	Bratislava, JezBratislava	P	P	P	P	P	P	P	N	N	P
LI	Canicola,HondUtrech IV	P	P	P	P	P	P	P	N	N	P
LI	Grippotyphosa, Andaman	P	P	P	P	P	P	P	N	N	P
LI	Hardjo, hardjoprajitino	P	P	P	P	P	P	P	N	N	P
LI	icterohemorrhagiaeM20	P	P	P	P	P	P	P	N	N	P
LI	Pomona, Pomona	P	P	P	P	P	P	P	P	N	P
LB	javanica, javanica	P	P	P	N	P	P	P	P	N	P
LB	hardjobovis NVSLS1343	P	P	P	N	N	P	P	P	N	P
LB	Hardjobovis B	P	P	P	N	N	P	P	P	N	P
LB	Tarassovi , perepelicin	P	P	P	N	N	P	P	P	N	P
LBF	patoc, patoc1	N	N	N	N	N	P	P	N	N	P

Key: P – Positive N – Negative LI – *Leptospira interrogans* LBF – *Leptospira biflexa*

LB – *Leptospira borgpetersenii*

6.2.2.2 Extraction of DNA from the samples.

Urine samples

Cattle urine samples collected from the three dairy farms stated above were processed By aliquoting 1.5ml each into centrifuge tubes. The urine samples were spun at 14,000 rpm for 1minute at 24^{o}C. The tube was removed from the centrifuge and the supernatant discarded. Additional 1.5ml of the urine sample was added to each tube and they were spun again at 14000 rpm for 1 minute at 24^{o}C. The supernatant was discarded and the sediment was retained for DNA extraction using the Mo Bio™ ultra clean DNA Extraction kit. The manufacturer's instructions were strictly adhered to. The extracted DNA from each sample, appropriately labeled was stored in sterile tubes at -20^{o}C.

Kidney samples

The kidney samples used for this test were from the same source as those used in the DFM and FAS tests.

About 1g of kidney was cut from the sample, dipped into alcohol and passed through the flame as described already. The kidney piece was then placed in a Petri dish and macerated by cutting it into fine shreds with the pair of scissors. The macerated tissue was put in a micro tube and processed for DNA extraction using Mo Bio™ tissue ultra clean DNA kit according to the manufacturer's instructions. The extracted DNA from each sample, appropriately labeled was stored in sterile tubes at -20^{o}C.

6.2.2.3. Conventional Polymerase Chain Reaction on extracted DNA samples

The conventional PCR was carried out on the extracted DNA from each sample. Nuclease-free water was used as the negative control while extracted DNA from a pure isolate of *Leptospira interrogans* serovar Icterohaemorrhagiae was used as positive control. Master mix (containing $MgCl_2$ at 1.5Mm, 0.2Mm each deoxynucleoside triphosphate (dATP, dTTP, dGTP and dUTP) and 1.OU of platinum Taq DNA polymerse) for the conventional PCR was stored in microtubes at -80^{o}C. The microtubes were removed from the freezer a few minutes before use to allow the contents to thaw at room temperature. The following were added to the master mix: 1µl each of forward and reverse primers and 5µl of each sample DNA. For the positive control, 5µl of the *L. interrogans* serovar Icterohaemorrhagiae DNA was added and for the negative control, 5µl of nuclease-free water was added. The total PCR reaction volume was 20 µl. The

microtubes were placed in a mini centrifuge and spun. The microtubes were then placed in the thermal cycler where template denaturation, primer annealing and primer extension took place. Denaturation involved 40 cycles at 94^{o}C for 30 seconds, annealing takes place as the temperature drops to 50^{o}C for 20 seconds after which the extension takes place at 72^{o}C for 2 minutes.The amplified products were analyzed by 1% agarose gel (stained with 5µl of ethidium bromide) electrophoresis. A 100bp ladder was used and the volume of amplicon used for electrophoresis was 5µl. The electrophoresis was run at 110 volts for 20-30 minutes. The result was visualized with a U-V transilluminator and the image was captured and printed with a Kodak camera connected to it.

6.2.2.4 Determination of rapidity of the PCR technique

Urine samples

The time required to carry out the procedure from the point of pipetting the first 1.5ml of urine into the centrifuge tube to the point of observing the results of the gel picture and determining the sizes of the DNA bands was determined. A clock and a stop clock were set and used for this. The time of commencement of the procedure and the time it was completed were recorded for each of the first ten samples and the mean value was used as the time taken to run a single test.

Kidney samples

The time required to carry out the procedure from the point of cutting the 0.1g piece of kidney sample for DNA extraction to the point of obtaining result was taken as described above for the urine samples.

6.2.2.5 Determination of the cost of PCR technique.

The fixed costs of the major equipment were calculated using their depreciation values. This was done by dividing the unit cost of the equipment by the lifespan of the equipment. The variable costs which are the cost of consumable materials were calculated.

This was used to calculate the operating cost per annum. (See Appendix V and VI)

6.3 RESULTS

6.3.1 Isolates and samples positive by PCR test.

Isolates

The result of the tests on the isolates showed that three sets of primers (1, 2, 3) amplified all pathogenic leptospires but did not amplify the non pathogenic *Leptospira biflexa.* Another three sets of primers (6, 7, and10) amplified all *Leptospira* serovars. Primer sets 8 and 14 amplified only *L. borgpetersenii* species and primer sets 4 and 5 amplified only *L. interrogans spp.* A summary of the results is given in Table 6.2.

Samples

Some of the samples from cattle urine were amplified by Primer 1 (Plate 6.1). Samples from a coyote, and some foxes were amplified by Primer 8 (Plate 6.2) and an opossum sample was amplified by Primer 14 (Plate 6.3).

Out of a total of 167 samples tested, 17 (10.2%) were positive. Whereas only 6 (6.70%) of the urine samples were positive, 11 (14.3%) of all the kidney samples were positive. Coyotes and foxes had the highest prevalence of 50%, followed by opossum (40%). Samples from other wildlife were negative (Table 6.3).

The highest percentage of positive samples (25.8%) for PCR was recorded in the wildlife kidneys as shown in Figure 6.1.

Table 6.3: Overall prevalence of leptospirosis as detected by PCR technique

Specimen	Species	No. tested	No. positive	% positive
Urine	Cattle (*Bos taurus*)	90	6	6.70
Kidney	Cattle (*Bos taurus*)	16	1	6.30
Kidney	Dogs (*Canis lupus familiaris*)	30	2	6.70
Kidney	Bob cats (*Lynx rufus*)	5	0	0
Kidney	Beavers (*Castor canadensis*)	2	0	0
Kidney	Raccoon (*Procyon lotor*)	7	0	0
Kidney	Coyotes (*Canis latrans*)	2	1	50.00
Kidney	Foxes (*Vulpes vulpes*)	10	5	50.00
Kidney	Opossums(*Didelphis virginiana*)	5	2	40.00
	SUB-TOTAL KIDNEY	77	11	14.3
	GRAND TOTAL	167	17	10.2

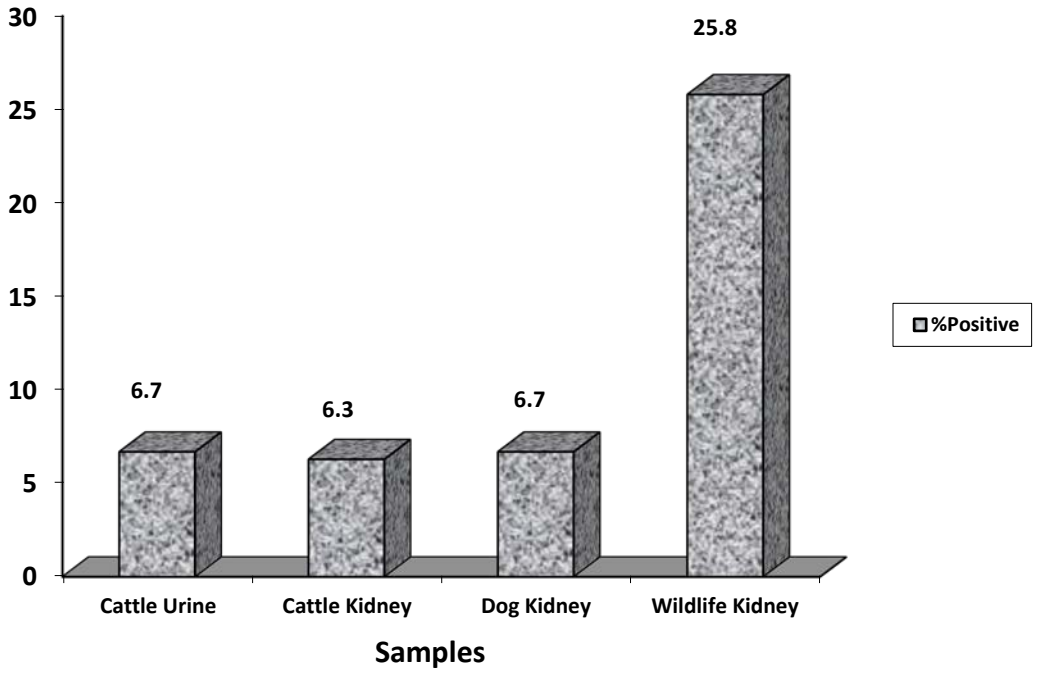


Fig.6.1: Percentage of samples detected positive by Polymerase chain reaction (PCR)

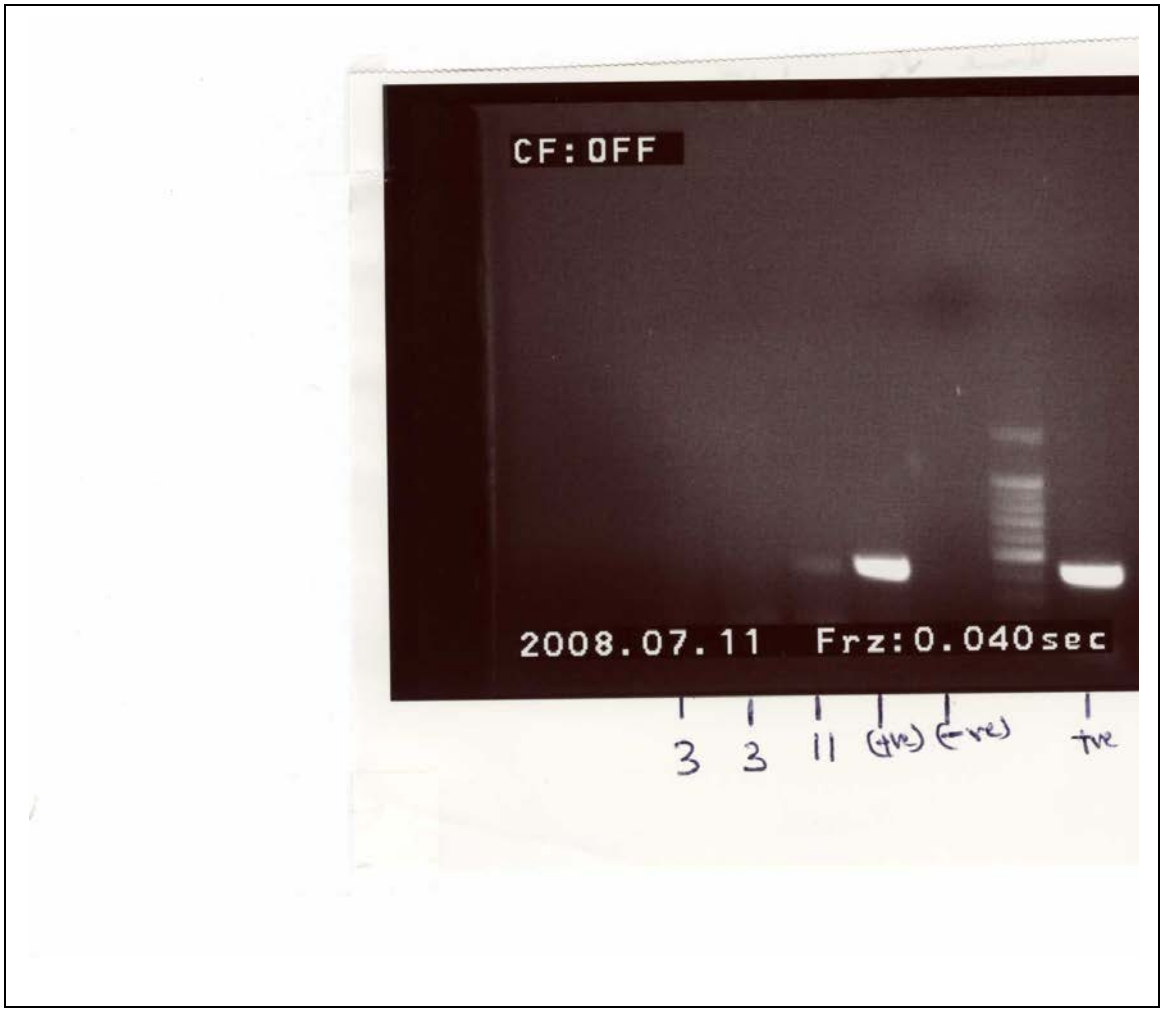


Plate 6.1 DNA extract of dairy urine samples of OH_3 and OH_{11} using primer LIP

Sample 3 (OH_3) showed negative result. Sample 11 (OH_{11}) showed faint positive result with expected product size of 423bp, same as positive control. Size marker = 100bp

Plate 6.2: Results of PCR on DNA extracts from wildlife kidney tissues using primer 737 and 1218

The following samples were positive were positive with expected band sizes of 482bp

Samples: 16======== Coyote (CY_2)

18======== Grey fox (GF_1)

20======== Grey fox (GF_3)

21======== Grey fox (GF_4)

22======== Grey fox (GF_5)

Size marker = 100bp ladder

Plate 6.3: Results of PCR on DNA extracts of wildlife kidney tissues using primer 1533 and 2

Lane 23 is positive showing same product size of 773bp as the positive control(+).
KEY:

Lane 23==========Opossum (O_1)

Size marker = 100bp ladder

6.3.2: Rapidity of the PCR technique

The test showed that it took an average of 304 minutes to run the PCR test on each sample.

6.3.3: Cost of operation of PCR technique per annum and cost per unit test.

Cost of fixed equipment/materials		₦563,370.00
Cost of consumables (variable cost)		₦559,797.10
Cost of labour		₦1,200,000.00
Total cost per annum		₦2,323,167.10
Cost per unit test		
Cost of total tests in a year		₦2,323,167.10
Cost of tests in a month	(Y/12)	₦2,323,167.10/12
		₦193,597.26
Cost of tests in a week	(M/4)	W =193,597.26/4
		₦48,399.32
Cost of tests in a working day	(W/5)	D = ₦48,399.32/5
		₦9679.86
Period of work in a day (9a.m.- 5p.m, 1hr. of lunch break is excluded)		T = 420 min.
Time required to run through one test		t = 305 min.
Number of tests done daily	(T/t)	d = 1.38tests
Cost per unit test	(D/d)	C = ₦ 9679.86/1.38
		₦7,014.39

6.4 DISCUSSION

The PCR technique had the least number of positive results among the three techniques evaluated. This might be due to the claim of Smythe *et al.* (2002) that test sensitivity depends on the ability of the primers to detect pathogenic species of the genus circulating in the population as well as the type of PCR adopted by the laboratory. It could be that the primers used for these tests were not able to detect the pathogenic species in the sampled environment. Consequently, it is pertinent for each laboratory to develop a library of primers that would be able to detect all the pathogenic species of leptospirae within its environ. In addition, regular survey, surveillance and monitoring should be done to keep abreast of the dynamic demographic changes that may be responsible for the introduction of new species in the same environment over time. Only few of the primer pairs for PCR detection of leptospires have been shown to amplify leptospiral DNA from veterinary clinical materials (Van Eys *et al.,* 1989; Zuerna *et al.,* 1995; Masri *et al.,* 1997; Taylor *et al.,* 1997; Wagenaar *et al,* 2000;). A possible reason for this may be the presence of inhibitors in clinical materials because the trial tests carried out on 'spiked' samples containing various serovars from cultures gave the expected positive results. In a similar test carried out by Bal *et al.* (1994), it was discovered that a disappointing 50% was missed by PCR. This was linked to the possibility that the number of leptospires in the samples was too small to be detected.

The operating cost of a PCR technique is ₦2,323167.1 and the cost per unit test is ₦7014.39. This is expensive and may not be affordable to the average farmer or pet owner in Nigeria.

CHAPTER SEVEN

7.0 COMPARISON OF THE THREE TECHNIQUES (DFM, FAS, PCR)

7.1 INTRODUCTION

Early diagnosis of leptospirosis is essential because prompt, specific treatment is important to ensure a favourable clinical outcome (Dey *et al*., 2007). The three techniques: DFM, FAS and PCR have been used at different times in various places by many workers (Arzouni *et al.*, 2002; Smythe *et al.*, 2002; Bolin, 2003) and their various advantages and disadvantages have been evaluated.

7.2 METHODS

7.2.1 Comparison of the relative sensitivity, relative specificity, accuracy and Kappa statistics of DFM to FAS, DFM to PCR and FAS to PCR.

This was calculated according to the EN ISO 16140 standard (Dey *et al*., 2007)
(See Appendix VIII, IX and X for formulae).

7.2.2 Test of relationship among the three techniques.

A correlation analysis was run to test for relationship among the three techniques using Pearson's coefficient.

7.2.3 Evaluation of the rapidity of the three diagnostic techniques.

The time taken to detect leptospires in samples using each of the DFM, FAS and PCR techniques were statistically compared. The Students't-test was used to determine if there were significant differences in the times.

7.2.4 Evaluation of the operating cost and cost per unit test of the three techniques

The operating cost per annum as well as the cost per unit test for each technique were calculated to determine the most affordable of the three techniques.

7.3 RESULTS

7.3.1 Relative sensitivity, relative specificity, accuracy and Kappa statistics.

The relative sensitivity, specificity and accuracy of DFM compared with FAS were 88.0%, 96.0%, 94.7%, respectively and compared with PCR were 64.7%, 73.5%, 72.5% respectively. The relative sensitivity, specificity and accuracy of PCR compared with FAS were 34.3%, 99% and 83.2% respectively. Kappa statistics showed perfect agreement (*k*=0.99) between DFM and FAS, DFM and PCR and between PCR and FAS

(Table 7.1)

7.3.2 Results of correlation analysis

This showed that there was a significant weak positive relationship existing between DFM and FAS. A non significant relationship existed between DFM and PCR while a significant positive relationship existed between FAS and PCR (Table 7.2).

7.3.3 Comparison of rapidity of the three techniques

Rapidity of the tests were 26.1 minutes, 120.0 minutes and 304.0 minutes per test for DFM, FAS and PCR respectively. The diagnostic time for each technique was significantly different from the others. Details are shown in Appendices VIII, IX and X.

7.3.4 Evaluation of cost per unit test for each technique

The cost per unit test for DFM, FAS and PCR were ₦744.46, ₦1974.68 and ₦7014.39 respectively (Table 7.3).

Table 7.1: Comparison of the relative sensitivity, relative specificity and accuracy of the three techniques

Tests	Relative sensitivity (%)	Relative specificity (%)	Accuracy (%)
DFM/FAS	88.0	96.0	94.7
DFM/PCR	64.7	73.5	72.5
PCR/FAS	34.3	99.0	83.2

Table 7.2: Correlation analysis showing the relationship among the three tests (DFM, FAS and PCR)

Correlations

		dfm	fas	pcr
dfm	Pearson Correlation	1	.215**	-.034
	Sig. (2-tailed)	.	.005	.660
	N	166	165	166
fas	Pearson Correlation	.215**	1	.241**
	Sig. (2-tailed)	.005	.	.002
	N	165	166	166
pcr	Pearson Correlation	-.034	.241**	1
	Sig. (2-tailed)	.660	.002	.
	N	166	166	167

**. Correlation is significant at the 0.01 level (2-tailed).

From the correlation test above, the P-value = 0.005 < 0.05 level of Significance, and Correlation coefficient = 0.215. This implies that there was a significant weak positive relationship existing between DFM and FAS test. While, a non significant relationship was found existing between DFM and PCR test i.e P-value = 0.660>0.05 level of Significance, though a negative weak relationship was found (correlation coefficient =-0.34),but it was not statistically significant.

A low significant positive relationship was found existing between FAS and PCR test, i.e P-value = 0.002 < 0.05 level of Significance. and Correlation coefficient = 0.241.

Table 7.3: Comparison of diagnostic time (rapidity) between DFM and FAS

Tests	Mean Time (Mins.)	Standard deviation	T-statistics	P-value
DFM	26.10	3.56	1.20	0.00
FAS	119.96	4.09		

RESULT = Diagnosis time through FAS is significantly higher than diagnosis time through DFM.

Table 7.4: Comparison of diagnostic time (rapidity) between DFM and PCR.

Tests	Mean time (min.)	Standard deviation	T-statistic	P-value
DFM	26.10	3.56	2.60	0.00
PCR	305.00	6.72		

RESULT = Diagnosis time through PCR is significantly higher than diagnosis time through DFM.

Table 7.5: Comparison of diagnostic time (rapidity) between FAS and PCR

Tests	Mean time (min.)	Standard deviation	T-statistics	P-value
FAS	119.96	4.09	1.70	0.00
PCR	305.00	6.72		

RESULT = Diagnosis time through PCR is significantly higher than diagnosis time through FAS.

Table 7.6: Cost per unit test for each technique

Tests	Maximum Number of Tests per annum	Operating Cost (₦) per annum	Cost (₦) per unit test
DFM	3840	2,858,714.29	744.46
FAS	960	1,895,692.5	1974.68
PCR	331	2,323,167.1	7014.39

7.4 Test of hypotheses

There is perfect agreement between the results obtained by dark-field microscopy and those obtained by fluorescent antibody staining.

This hypothesis has been tested to be true. The results of this work show that the relative sensitivity, relative specificity and accuracy between the DFM and FAS were 88.0%, 96.0% and 94.7%. The Kappa statistic value K=0.99 also shows there is perfect agreement.

There is perfect agreement between the results obtained by dark-field microscopy and those obtained by polymerase chain reaction.

This hypothesis has been tested to be true. The results of this work show that the relative sensitivity, relative specificity and accuracy between the DFM and PCR were 64.7%, 73.5% and 72.5% respectively. The Kappa statistic value K= 0.99 also shows there is perfect agreement.

There is perfect agreement between the results obtained by fluorescent antibody staining and those obtained by polymerase chain reaction. The hypothesis has been tested to be true. The results of this work show that the relative sensitivity, relative specificity and accuracy between FAS and PCR were 34.3%, 99% and 83.2% respectively. The Kappa statistic value K= 0.99 also shows perfect agreement.

Dark-field microscopy is not as rapid as fluorescent antibody staining for leptospirosis diagnosis.

This hypothesis has been tested not true because the time taken for diagnosis by FAS was an average of 120 minutes while for DFM, the average time was 26 minutes (Table 7.3).

Dark-field microscopy is not as rapid as polymerase chain reaction for leptospirosis diagnosis. This hypothesis has been tested not true because the time taken for diagnosis by PCR was an average of 305 minutes while for DFM it was an average of 26 minutes (Table 7.4).

Fluorescent antibody staining is not as rapid as polymerase chain reaction for leptospirosis diagnosis. This hypothesis has been tested not true because an average of time of 120 minutes was required for FAS while an average time of 305 minutes was required for PCR (Table 7.5).

Dark-field microscopy costs less per unit than either fluorescent antibody staining or polymerase chain reaction for leptospirosis.

This hypothesis has been tested to be true because the cost per unit test for DFM (₦744.46) was a lot cheaper than the cost per unit test for FAS (₦1974.68) and PCR (₦7014.39) respectively.

7.5 Discussion

As previously stated by various authors quoted in this work (Dey *et al.,* 2007; Centre for Food Security and Public Health, 2005), leptospirosis has often been misdiagnosed because the presenting signs are similar to those seen in a lot of other disease conditions. Outbreaks of leptospirosis have been reported when people were exposed to flood waters contaminated with leptospires (WHO, 2000; Seghal *et al.,* 2001). Also, leptospirosis has been recognized as a possible sequel to natural disasters and floods because people and animals are exposed to wet environments for a long period of time. Currently, there is no system in place to monitor the occurrence and prevalence of leptospirosis in Nigeria, even though Nigeria has experienced some catastrophic floodings in recent times. This is probably due to the fact that there is no standard diagnostic technique available for use and there are no trained and skilled personnel to carry out the necessary tests. Consequently, developing countries like Nigeria where leptospirosis is endemic, have no specific control programme in operation and surveillance is often lacking or incomplete. Therefore outbreaks of the disease continue to occur in Nigeria and even an estimate of the disease burden is not available.

The bane of most developing countries like Nigeria is lack of sufficient funds. This automatically leads to inability to put the necessary and required infrastructures in place. In addition, the required man-power and skill acquisition for demand driven and result oriented research are predicated on availability of funds.

The result of this research has clearly shown that screening for leptospirosis and diagnosis of this disease could be done and is quite affordable in Nigeria with the use of the dark field microscope CytoViva™. The skill required to operate the equipment is minimal and those already trained as laboratory or medical technologists or microbiologists will need minimal training to operate it. The result of the evaluation of this technique shows that the sensitivity, specificity and accuracy compare favourably with those of the FAS. The accepted method for the confirmation of leptospirosis is culture. Previous works have reported that FAS compares favorably with culture technique (White *et al*., 1961; Boulanger and Robertson, 1961; Bolin *et al.,* 1989). The DFM has the advantage of being applicable for early diagnosis before the antibodies are

formed and it is also a very rapid test. This will make prompt and accurate treatment a reality thus eliminating the problem of drug (antibiotic) abuse. The rapidity of the test allows for more samples to be processed within a specified period of time compared to the other tests evaluated. This is also an advantage that makes it possible to use this technique for screening (large sample size) as well as diagnosis. The problem of ensuring that primer sets for the serovars in circulation are available as required for accurate PCR result is eliminated. The money, space and time as well as skill acquisition required for the other techniques evaluated (FAS and PCR) far exceed the requirement for DFM. Further confirmation of the test result if so required may be carried out using any of the other methods like culturing or serology.

7.6 Conclusion

This work has shown that the morbidity rate of canine leptospirosis in Ibadan was 47.0% and further confirmed the endemic nature of the disease in the sampled population. This information is vital because of the public health implication as leptospirosis is an infectious zoonosis that affects almost all species of animals. This work has also showed that there is agreement between the results obtained by using the DFM (cytoviva™) and the previously established and accepted method, FAS. The relative sensitivity, relative specificity and accuracy of DFM to FAS were 88.0%, 96.0% and 94.7% respectively.

It was concluded in this work that the DFM (cytoviva™) was a more rapid test (26 min.) than the FAS (120min.) and PCR (305min.) respectively.It was also established that the DFM was a cheaper test, costing ₦744.46 per unit test, than the FAS (₦1974.68) and the PCR (₦7014.39) .

7.7 Recommendations

a) The recommended method for Leptospirosis diagnosis in Nigeria is the Dark field microscopy (cytoviva™). This is because it is sensitive, accurate, rapid and cheap, costing less per unit test than the other two techniques evaluated.

b) Samples of choice for diagnosis are urine in live animals and kidney tissues for post mortem.

c) Dogs should be screened and vaccinated regularly against canine leptospirosis.

d) Whenever there is flooding, humans, pets and livestock should be screened for leptospirosis.

e) Leptospirosis reference laboratories should be established in each geopolitical zone of the nation.

7.8 Contribution to knowledge.

This work has been able to determine the current prevalence of canine leptospirosis in Ibadan.

Whereas few methods had been previously used for diagnosis of leptospirosis in Nigeria, there is no known evaluation or comparison of these methods. This work has been able to fill the knowledge gap. The work has effectively evaluated and compared the sensitivity, accuracy, rapidity and cost of DFM, FAS and PCR.

This work has shown that the DFM is equally sensitive, more rapid and cheaper than the FAS and PCR.

The knowledge derived from this work for adequate diagnosis would enable the nation to have a data base on the current status of leptospirosis in Nigeria for research , monitoring, surveillance and control purposes.

This work has given insight to the opportunity of moving Nigeria beyond the level of leptospirosis diagnosis based only on clinical signs and post – mortem reports to the level of confirmatory diagnosis on live animals.

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APPENDICES

I. Cost of Fixed Equipment for DFM

Items	Unit Cost (N)	Span (years)	Depreciation Value (N)
DFM (cytoviva)	5,250,000	10	525,000
Stomacher	292,500	10	29,250
Inoculating hood	180,000	10	18,000
Centrifuge	40,000	10	4,000
Electrical pipette pump	28,600	10	2,860
Bunsen Burner	45,000	5	9,000
Clock	1,500	5	300
Stop clock	3000	5	600
Micro pipette 100ul	2,500	10	250
Micro pipette 1000ul	24,000	4	6,000
Racks for microtubes	30,000	5	700
Slide rack	3,500	5	500
Beaker (100ml)	2,500	5	500
Total	5,892,100		596,960

II Cost of Consumable Items (Variables) for DFM per Annum

Items	Quantity	Cost (₦)
Centrifuge tubes	90 packs	229,252.50
Cover slips	45 packs	15,505.00
100 glass pipettes	100 pieces	85,000.00
PBS 1000ml	1000ml	3,975.00
Paper towels	100 packs	100,000.00
Alcohol	2000 ml	2,000.00
Glass slides	62 packs	15,382.00
6 pairs of forceps	6 pairs	10,500.00
6 pairs of scissors	6 pairs	2,400.00
Micro tubes	90 packs	229252.50
Pipette tips	5 packets	18,000.00
Markers	6 packets	7,500.00
Stomacher bags	20 packs.	800,000
Gloves	15 packs	15000
Labour	1 worker	727,987.29
Total		2,261,754.29

III Cost of Consumable Items (Variables) for FAS per Annum

Items	Quantity	Cost (N)
Centrifuge tubes	20 packs	49,680.00
Cover slips	10 packs	3,360.00
PBS 3000ml	3000ml	11,925.00
Alcohol	2000 ml	2,000.00
Fluorescent slides	20 packs	13,440.00
2 pairs of forceps	2 pairs	3,500.00
2 pairs of scissors	2 pairs	800.00
Beaker (100ml)	4	2,000
Micro tubes	20 packs	49,680.00
Pipette tips	20 packs	4,000.00
Gloves	10 packs	15000
Paper towels	15 packs	15000
Disposable inoculating loop	1000pcs.	23,985
Mountant	30 bottles	255,000.00
Markers		7,500.00
Labour	1 worker	900,000.00
Acetone	1 litre	9,864.00
FITC-lepto conjugate		50,000
Stomacher bags	5packs	200,000
Petri dishes		2,998.50
Total		1,619,732.5

IV Cost of Fixed Equipment for FAS

Items	Unit Cost (N)	Span (years)	Depreciation Value (N)
Fluorescent Microscope	1,600,000	10	160,000
Stomacher	292,500	10	29,250
Inoculating hood	180,000	10	18,000
Centrifuge machine	40,000	10	4,000
Electrical pipette pump	28,600	10	2,860
Bunsen Burner	2,500	5	9,000
Clock	1,500	5	300
Stop clock	3,000	5	600
Micro pipette 100ul	24,000	10	250
Micro pipette 1000ul	30,000	4	6,000
Racks for microtubes	3,500	5	700
Slide rack	2,500	5	500
Incubator	350,000	10	35,000
Refrigerator	95,000	10	9,500
Total	2,653,100		275,960

V Cost of consumable items for PCR per annum

Items	Quantity	Cost (N)
Centrifuge tubes	7 packs	`17,439.80
Mo Bio™ Ultra clean tissue kit	4 kits	45,000.00
PBS	1000ml	3,975.00
Alcohol	2000 ml	2,000.00
Ethidium bromide	15 vials	255,000.00
Forceps	6 pairs	10,500.00
Scissors	6 pairs	2,400.00
Master mix	7 packs	17,439.8.
Micro tubes	20 packs	49,680.00
Pipette tips	20 packs	4,000.00
Gloves	15 packs	30,000.00
Paper towels	30 packs	30,000.00
PCR tubes	5 packs	25,000.00
Ladder	15 vials	25,000.00
Markers		7,500.00
Labour	1 worker	1,200,000.00
Acetone	1 litre	9,864.00
Primers		10,000
TAE	4 bottles	12,000
Petri dishes		2,998.50
Total		1,759,797.1

VI Cost of Fixed Equipment for PCR

Items	Unit Cost (N)	Span (years)	Depreciation Value (N)
DNA Work station	350,900	10	35,090.00
Gradient Cycler	2,500,000	10	250,000
Gel documentation and analysis system	600,000	10	60,000
Centrifuge machine	40,000	10	4,000
Micro pipette 10µl	20,000	4	5,000
Bunsen Burner	2,500	5	9,000
Clock	1,500	5	300
Stop clock	3,000	5	600
Micro pipette 100ul	24,000	10	250
Micro pipette 1000ul	30,000	4	6,000
Racks for microtubes	3,500	5	700
Microwave oven	24,000	10	2,400
Gel trays	67,500	10	6,750
Refrigerator	95,000	10	9,500
Freezer (-80^{o}C)	490,000	10	49,000
Metler weighing balance	464,400	10	46,440
Vortex mixer	58,500	10	5,850
Mini centrifuge	54,900	10	5,490
Freezer (-20^{o}C)	490,000	10	49,000
Total	5,319,700.00		563,370.00

VII TEST B Hypothesis = There is no difference between diagnosis time through kidney and urine specimens using FAS method.

. ttest fas, by(specimen)

Two-sample t test with equal variances

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      20      115.15    .0819178    .3663475    114.9785    115.3215
       1 |      31    123.0645    .2779939    1.547805    122.4968    123.6323
---------+--------------------------------------------------------------------
combined |      51    119.9608     .572549    4.088818    118.8108    121.1108
---------+--------------------------------------------------------------------
    diff |           -7.914516    .3534588               -8.624818   -7.204214
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t = -22.3916
Ho: diff = 0                                     degrees of freedom =       49

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0000         Pr(|T| > |t|) = 0.0000          Pr(T > t) = 1.0000
```

RESULT = Diagnosis time by FAS method through kidney is significantly higher than time through urine specimen.

VIII TEST D Hypothesis = There is no difference between diagnosis time using DFM and FAS methods.

. ttest DFM == FAS, unpaired

Two-sample t test with equal variances

Variable | Obs Mean Std. Err. Std. Dev. [95% Conf. Interval]

---+---

dfmfas~r | 51 26.05882 .5020833 3.585592 25.05036 27.06729

fas | 51 119.9608 .572549 4.088818 118.8108 121.1108

---+---

combined | 102 73.0098 4.687134 47.33774 63.71179 82.30782

---+---

diff | -93.90196 .7615117 -95.41278 -92.39114

---

diff = mean(DFM) - mean(FAS) t = -1.2e+02

Ho: diff = 0 degrees of freedom = 100

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0

Pr(T < t) = 0.0000 Pr(|T| > |t|) = 0.0000 Pr(T > t) = 1.0000

RESULT = Diagnosis time through FAS is significantly higher than diagnosis time through DFM.

IX TEST E Hypothesis = There is no difference between diagnosis time using DFM and PCR methods.

. ttest dfm == pcr, unpaired

Two-sample t test with equal variances

Variable | Obs Mean Std. Err. Std. Dev. [95% Conf. Interval]

---+---

dfm | 51 26.05882 .5020833 3.585592 25.05036 27.06729

pcr | 51 304.9804 .9403755 6.715624 303.0916 306.8692

---+---

combined | 102 165.5196 13.887 140.2518 137.9715 193.0677

---+---

diff | -278.9216 1.066018 -281.0365 -276.8066

---

diff = mean(dfm) - mean(pcr) t = -2.6e+02

Ho: diff = 0 degrees of freedom = 100

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0

Pr(T < t) = 0.0000 Pr(|T| > |t|) = 0.0000 Pr(T > t) = 1.0000

RESULT = Diagnosis time through PCR is significantly higher than diagnosis time through DFM.

X TEST F Hypothesis = There is no difference between diagnosis time using FAS and PCR methods.

. ttest fas == pcr, unpaired

Two-sample t test with equal variances

Variable | Obs Mean Std. Err. Std. Dev. [95% Conf. Interval]

---+---

fas | 51 119.9608 .572549 4.088818 118.8108 121.1108

pcr | 51 304.9804 .9403755 6.715624 303.0916 306.8692

---+---

combined | 102 212.4706 9.221352 93.13109 194.1779 230.7633

---+---

diff | -185.0196 1.100962 -187.2039 -182.8353

---

diff = mean(fas) - mean(pcr) t = -1.7e+02

Ho: diff = 0 degrees of freedom = 100

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0

Pr(T < t) = 0.0000 Pr(|T| > |t|) = 0.0000 Pr(T > t) = 1.0000

RESULT = Diagnosis time through PCR is significantly higher than diagnosis time through FAS.

XI Calculation of the relative sensitivity, relative specificity and accuracy of DFM, FAS, and PCR in the detection of leptospires.

The relative sensitivity, relative specificity and accuracy of DFM were calculated in comparison to FAS according to the EN ISO 16140 standard.

A modification of the formula stated by Dey *et al*. (2007) was used for calculation:

Sensitivity.

a/ (a+c) x 100

a number found positive by DFM and FAS

c number found positive by DFM and negative by FAS

Specificity.

d/(b+d) x 100

d number found negative by both DFM and FAS

b number found negative by DFM and positive by FAS

Accuracy.

(a+d)/ (a+b+c+d) x100

k (Kappa statistics) (a+d –P)/ 1-P

P (a+b)(a+c) + (c+d) (b+d)

P Probability

a number of samples found positive by both DFM and FAS

b number of samples found positive by DFM and negative by FAS

c number of samples found negative by DFM and positive by FAS

d number of samples found negative by both DFM and FAS.

The same formula was used to calculate the relative sensitivity, relative specificity and accuracy of DFM to PCR and of FAS to PCR.

XII Pictures of examples of wildlife sampled

Beaver

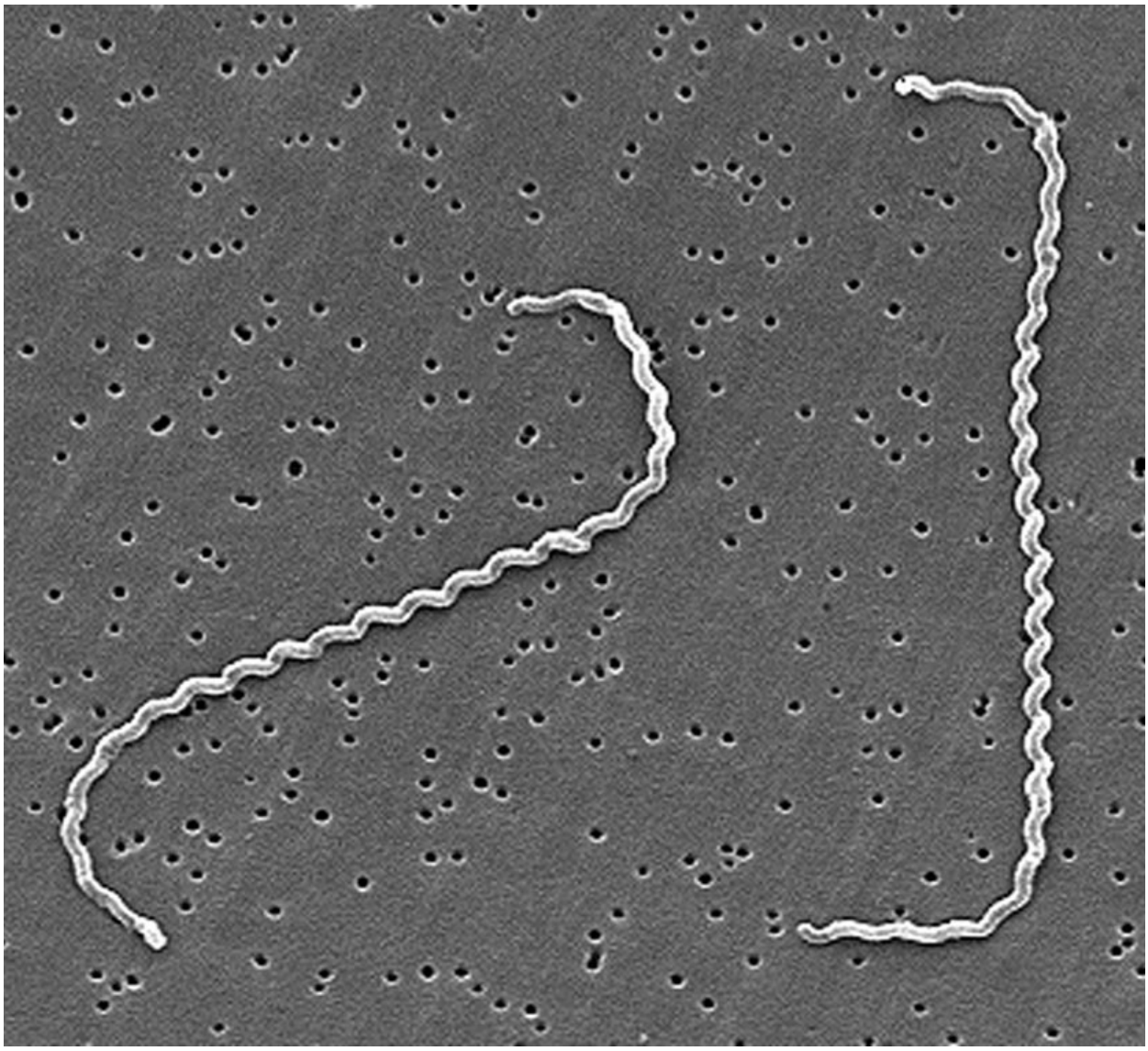
coyote

REDFOX

opossum

RACOON

XIII Electron micrograph of leptospires showing helical structure and curved (hooked) ends (original magnification × 60,000).



Source: Weyant *et al.,*1999. Manual of Clinical Microbiology