EPIDEMIOLOGICAL AND BACTERIOLOGICAL STUDIES ON ANIMAL AND HUMAN LEPTOSPIROSIS IN PLATEAU STATE, NIGERIA.

BY

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DECLARATION

I hereby declare that I carried out the work reported in this thesis at the Veterinary Research Institute, Vom and in the Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine under the supervision of Professor P.B. Addo, Dr. A.A. Adesiyun and Dr. C.S.S. Bello, and in Belfast under the supervision of Dr. W.A. Ellis.

The work of other investigators is duly acknowledged and referred to. No part of this has been submitted elsewhere for a degree.

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CERTIFICATION

This thesis entitled:- "Epidemiological and bacteriological studies on animal and human leptospirosis in Plateau State, Nigeria" by Anthony Okay Ezeh meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my family particularly to Ify and Uche who voluntarily refused artificial baby milk in order to reduce the financial burden imposed by the project on the family.

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ABSTRACT

Leptospirosis is an infectious and contagious disease of animals and man caused by spiral-shaped organisms belonging to the genus Leptospira. Although it is known that leptospirosis is prevalent in the tropical and subtropical areas of the world, few studies on the disease have been reported in Nigeria. The present study was designed to determine the prevalence rate of leptospiral antibodies in voluntary blood donors and cattle in the 14 local government areas of Plateau State; to attempt isolation of leptospires from bovine and rodent kidneys; to characterize all such isolates and determine their pathogenicity through experimental studies.

Leptospiral antibody titre of 1:100 or more was detected in 14.4 per cent of 1,537 cattle screened in the 14 local government areas of Plateau State using 13 live serovars in a modified microscopic agglutination test. Of the 222 animals that were sero-positive, antibodies to serovar hardjo was the most prevalent accounting for 35.6 per cent. The serological prevalence rates of bovine leptospirosis ranging from 7.9 per cent to 28.0 per cent in the different local government areas differed significantly ($P < 0.05$; X^2).

Six leptospiral strains belonging to Sejroe and Pyrogenes serogroups were isolated from 525 bovine kidneys examined bacteriologically. In a similar examination of

502 rodents, no leptospire was isolated but 44.8 per cent of 105 trapped rodents tested were serologically positive.

In a serological examination of serum samples obtained from 710 voluntary blood donors in the study area 18.0 per cent were positive for leptospirosis. Of the 128 sero-positive individuals, serovar hardjo was the most common with a prevalence rate of 21.9 per cent. There was no statistical significant difference in the prevalence rate of leptospirosis in the different local government areas ($P > 0.05$; X^2). Abattoir workers with a prevalence rate of 29.5 per cent were occupationally at risk of contracting leptospirosis and about 39.0 per cent of all pyrexic patients examined were serologically positive. A variant strain of Hardjoprajitno was isolated from the mid-stream urine of an abattoir worker.

Cross-agglutinin absorption tests showed that five of the bovine isolates were identical and they belonged to Pyrogenes serogroup. The sixth bovine and the only human isolates were similarly identified as serovar hardjo, genotypes hardjobovis and hardjoprajitno respectively. The isolation of these two hardjo genotypes is being documented for the first time in Nigeria and all the six bovine isolates exhibited biological properties consistent with pathogenic leptospires. The restriction endonuclease analysis patterns showed the uniqueness of the human isolate in having an extra band. Also, the restriction patterns of the bovine isolates were different from those of other reference serovars in the Pyrogenes serogroup.

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ABBREVIATIONS

°C	degree Celsius (centigrade)
CFT	complement fixation test
ELISA	enzyme-linked immunosorbent assay
EMJH	Ellinghausen-McCullough-Johnson-Harris medium
et al.	and others
FA	fluorescent antibody
H and E	haematoxylin and eosin
ip	intraperitoneally
kg	kilogram
MAT	microscopic agglutination test
mg	milligram
µg	microgram
µm	micrometre
ml	millilitre

Chapter 1

INTRODUCTION

Leptospirosis is an infectious and contagious disease of man and animals caused by spiral-shaped bacterium belonging to the genus Leptospira. Man is an accidental host that becomes infected through occupational exposures, through invasion of the wild environment and through contact with infected pets (Hanson, 1982). Occupational groups like veterinarians, abattoir workers, sewage workers, miners, livestock owners and attendants, farmers and dairy workers have been reported to be at high risk. The pathogenic species, Leptospira interrogans consists of approximately 180 serovars arranged within 19 serogroups according to their antigenic characteristics (Johnson and Faine, 1984).

Although leptospirosis is enzootic in nature, only a particular proportion of the pathogenic serovars is endemic in any particular region or country (Turner, 1967). In an area where leptospirosis is endemic, each infecting serovar tends to be maintained in specific maintenance hosts. Thus if the serovar causing human infection can be identified, the likely source of infection can often

be determined (Blackmore and Schollum, 1980). With appropriate host exposure and susceptibility, any of the pathogenic leptospires can cause varying degrees of illness in man and animals. The nature of the disease varies greatly with the host and the infecting serovar. Wildlife, particularly rodents are the major reservoirs of infection although cattle have been identified as the main maintenance hosts for serovar hardjo (Ellis et al., 1981).

The presence of carriers in a moist environment and their numbers as well as contact with livestock and man are important factors in the epidemiology of leptospirosis. The disease has had major impact on the health of man, domestic animals and cattle industry in the tropics and relatively little is known of leptospirae in African livestock (Amatredjo and Campbell, 1975). However, leptospires have been found in most African countries where they have been sought for. Published reports about leptospirosis in North, Central and East Africa abound but the same cannot be said of West and other parts of Africa. In Nigeria, very few studies on leptospirosis have been carried out although it is known that the disease is prevalent in the tropical and subtropical areas of the world. In a serosurvey, Diallo (1978) reported

that the incidence of human leptospirosis is about the same as in cattle in the eight northern states of Nigeria.

In a sustained effort to improve animal protein intake in Nigeria, various government agencies, individuals and companies imported many animal breeds from different parts of the world to upgrade the indigenous stock without screening for leptospirosis. As a result, many exotic leptospiral serovars are constantly brought in to complicate the epidemiological data of leptospirosis in Nigeria. Also there is some lack of awareness on the part of clinicians and veterinarians and pyrexia of unknown origin (PUO) is poorly investigated and managed. Consequently pyrexic patients with similar symptoms are usually erroneously treated for malaria because it is the commonest cause of fever in Nigeria. The climatic and ecological environment of Nigeria favour the growth and transmission of leptospires. Unlike most other parts of Nigeria, Plateau State has optimal conditions suitable for the survival and maintenance of leptospires throughout the year and therefore was chosen for this pilot study. In some rural populations like in most African countries, the villagers live in close proximity to their pet

animals and small ruminants which may be carriers of leptospirosis. And in arid areas of the country, man shares his water source with wild rodents, migrating cattle, sheep, goats and dogs during the dry season.

It is known from the limited studies available that leptospirosis exists in Nigeria yet little attention is paid to the disease either by human doctors or veterinarians possibly because other tropical diseases with similar signs and symptoms abound. A further intensive search for leptospirosis and its aetiologic agent in man, cattle and rodents is therefore necessary. The objectives of this study were:

(i) to determine the prevalence of leptospiral antibodies in voluntary donor individuals and cattle herds from the 14 local government areas of Plateau State;

(ii) to attempt isolation of leptospires from bovine and rodent kidneys;

(iii) to characterize all leptospiral isolates and conduct experimental studies to determine their pathogenicity.

Chapter 2

LITERATURE REVIEW

Leptospirosis was first described in 1886 by Adolf Weil (Alston and Broom, 1958) and the causative organism was successfully cultivated and named Spirochaeta icterohaemorrhagica (Inada et al., 1916). Stimson (1907) had demonstrated the same organism histologically in a patient's kidney. After the description of the disease in man, rodents (especially house and ditch rats) were implicated as the reservoir of icterohaemorrhagiae and Noguchi (1917) isolated the leptospire in a Norwagian rat. Other pioneer workers like Stokes and Ryle (1916), Costa and Troisier (1916) in Europe described different aspects of leptospirosis. In Germany, Huberner and Reiter (1915), Uhlenhuth and Fromme (1919) isolated and named the causative organism of leptospirosis as Spirochaeta nodosa and Spirochaeta icterogenes respectively. In England, Coles (1918) described the minute structure of Spirochaeta icterochaemorrhagiae in the common rat while Ido et al. (1918) implicated Spirochaeta hebdomadis as the causative organism of seven-day fever (nanukayami).

Between 1918 and 1919, Noguchi studied various aspects of Spirochaeta icterohaemorrhagiae including morphological characteristics, nomenclature, its survival in nature, the intermediate host and the acquired immunity of guinea pigs against S. icterohaemorrhagiae after inoculation with blood of yellow fever patients. The prevalence of icterohaemorrhagiae was studied in Brazil by Smillie (1920). Hayman and Lynch (1927) reviewed the state of spirochaetal jaundice and Alexander (1960) regarded canicola, icterohaemorrhagiae and grippotyphosa as the most prevalent serovars in the United States of America. Gaumont (1966) regarded icterohaemorrhagiae as the most prevalent serovar in France while Turner (1967) regarded hebdomadis, icterohaemorrhagiae and canicola as the predominant serovars in Great Britain. In Israel, serovars grippotyphosa and ballum were isolated from hedgehogs and rats (van der Hoeden, 1964).

2.1 Leptospirosis in Africa

In Africa, the study of leptospirosis was not vigorously pursued until 1946 when Van Riel in Zaire carried out in-depth studies on the disease. He

isolated many leptospiral serovars from man, rodents and water. He concluded that icterohaemorrhagiae, grippotyphosa, hebdomadis and butembo were the most prevalent serovars in Zaire. Earlier, Bordes and Rivoalen (1938) stated that leptospirosis was rare in North Africa but Mailloux and Kolochine-Erber (1961) reported proven cases of leptospirosis in Morocco, Tunisia, Algeria, Madagascar and South Africa. It would appear at this time that the resurgence of renewed interest on leptospirosis was due to the concurrent presence of febrile illnesses like yellow fever, relapsing fever, black water fever and infectious epidemic jaundice. According to Schuffner (1941) the main reason for leptospirosis research in Africa was the occurrence of both yellow fever and leptospirosis because of the similarities of their clinical presentation. The first proven case of leptospirosis due to L. icterohaemorrhagiae in Morocco was described by Couzi and Gaud (1944). Blanc et al. (1961) and Monlay (1962) described varying degrees of leptospirosis and asserted that Canicola, Icterohaemorrhagiae, Grippotyphosa and Australis serogroups were the most prevalent leptospires in Morocco. Mailloux (1965) also tested 140 patients

serologically and concluded that Australis, Icterohaemorrhagiae and Canicola serogroups were predominant.

In Algeria, Mailloux and Kolochine-Erber (1967) detected antibodies to icterohaemorrhagiae and australis in patients. Benhamou et al. (1955) had earlier described leptospirosis in a 30-year old Algerian with acute fever, meningitis and miliary lesions in the lungs, caused by Leptospira canicola. In Tunisia, an epidemic of icterohaemorrhagiae occurred in water - carriers in a mosque (Coursieres-Berthezene et al., 1949). Reports of leptospirosis in Egypt are scanty but L. canicola was isolated from a dog (Marompot and Barsoum, 1972). Spirochaetes isolated from patients suffering from infectious jaundice in Sudan were considered non-pathogenic because the clinical picture was mild and different from classical Weil's disease (Whitehead and Crough, 1926). In a serological examination of 758 sera from domestic and wild animals, Moch et al. (1975) reported that L. butembo was the predominant serovar in dogs but in camels, L. grippotyphosa was predominant.

In Kenya, Burdin et al. (1958) described an epidemic of leptospirosis due to L. grippotyphosa in

livestock and the first proven human cases of acute leptospirosis were reported by de Geus et al. (1969). Piercy (1951) described four dogs with clinical and pathological signs of leptospirosis. Ball (1966) isolated four strains of leptospires belonging to the Icterohaemorrhagiae and Grippotyphosa serogroups from gerbils in Kenya and suggested that cattle served as maintenance host for serogroup Grippotyphosa. Van Riel (1939) observed in Zaire that leptospirosis was occupation - associated and that it was more prevalent in males and the majority of cases occurred during the rainy season. Van Riel and Van Riel (1956) in an extensive and systematic study of leptospirosis reported that Icterohaemorrhagiae, Grippotyphosa, Bataviae, Hebdomadis and Butembo were the most prevalent serogroups in Zaire. Blanchard and Lefrou (1922) succeeded in transmitting leptospirosis from a patient's blood to guinea pigs. Graft (1965) examined 505 human sera in Zimbabwe and found only one sample positive but failed to isolate any leptospire from rats. Feresu (1988) examined 2,382 bovine sera at a dilution of 1:100 and found 27 per cent prevalence of leptospirosis in Zimbabwe. In South Africa, icterohaemorrhagiae was identified as the major serovar responsible for human leptospirosis (Newman

and Cohen, 1962; Thomson, 1964; Klopper, 1969). Andre (1955) and Gauthier et al. (1969) reported that icterohaemorrhagiae was the predominant serovar responsible for acute leptospirosis in the Reunion in South Africa.

Reference to leptospirosis in West African countries in the past was limited to case reports. In Senegal, Payet et al. (1966) diagnosed four acute cases of leptospirosis caused by L. tarassovi and L. icterohaemorrhagiae. Sankale et al. (1974) reported 6.4 per cent serologically confirmed cases of leptospirosis and isolated L. icterohaemorrhagiae from a rat (Cricetomys gambianus). Earlier, Goez (1933) noted that 17 per cent of the Africans from Senegal, Mali and Ivory Coast were serologically positive for L. icterohaemorrhagiae. Using the macroscopic agglutination test, Rothstein (1964) in Ghana examined 398 sera from suspected cases of infectious hepatitis and yellow fever and reported high prevalence of leptospiral antibodies. Kinebuchi and Afoakwa (1973) recorded 21 per cent seroreactors among 99 suspected cases of infectious hepatitis using the microscopic agglutination test. Hogerzeil et al. (1986a) reported 33 per cent prevalence from 460 serum samples obtained from rural Ghana. In the same area, Hogerzeil et al. (1986b) detected

confirmed leptospirosis in four out of 88 suspected cases of acute leptospirosis among hospital patients and only one out of 102 patients with jaundice. They concluded that most cases of acute leptospirosis in rural Ghana did not have reason for seeking hospital care. Fry (1967) reported that leptospirosis was endemic in Western Cameroun where he observed that 25 per cent of 200 pyrexic patients suffered from leptospirosis. Earlier, Graf (1936) described a male European patient with the classical picture of Weil's disease. He demonstrated leptospires in the patient's urinary deposit and transmitted the disease to a guinea pig.

2.2 Leptospirosis in Nigeria

In Nigeria, Connal (1923) isolated a spirochaete from the blood of a European patient suffering from black water fever and the organism was non-pathogenic for animals. A case of leptospirosis was reported in a patient suspected to be suffering from yellow fever in southern Nigeria (Leach and Canon, 1943). Collard (1962) demonstrated L. canicola antibodies in 4.5 per cent of human population in Katsina while Turner et al. (1975) tested 955 cattle

serologically in Kaduna, Kano, Gongola and Sokoto States and found 48 per cent positive. Holland et al. (1977) reported 72 per cent prevalence in sheep, goats and cattle from Plateau, Kano and Kaduna States of Nigeria. Diallo (1978) detected 11.1 per cent and 11.9 per cent serological prevalence of leptospirosis in human and cattle respectively in the eight northern states of Nigeria. Also in a bacteriological survey of leptospirosis in Zaria, Nigeria, Diallo and Dennis (1982) recovered eight leptospires from 252 trapped field rats and five isolates from 74 bovine kidneys. The serovars identified were australis, ballum and pyrogenes, but six of the isolates were not identified. Ezeh et al. (1987) examined 850 bovine serum samples from four cattle herds in Plateau State and reported 22.9 per cent prevalence of leptospirosis.

2.3 General Characteristics of the Genus Leptospira

The genus Leptospira consists of a group of morphologically distinct, flexible helicoidal organisms of the Order Spirochaetales (Noguchi, 1918). The cells measure 0.1µm in diameter and six to over 12µm in length. The organism is tightly coiled

on its long axis. One or both ends of the cell are typically hooked especially in liquid environment although on rare occasions the ends may be straight. Electronmicroscopic studies have revealed an axial filament running the entire length of the organism (Simpson and White, 1961). All species of Leptospira have two periplasmic flagella and the basal bodies of these periplasmic flagella are similar to those of the flagella of Gram-negative bacteria.

Leptospires are highly motile and the characteristic movements appear as an alternating rotation around the long axis and translation in the direction of the unhooked cell end. The unique motility of leptospires, combined with their narrow diameter, flexibility and shape allow them to pass through 0.1 - 0.45µm pore size membrane filters and to migrate within media solidified with up to 1 per cent agar (Faine, 1982). In environments of high viscosity, flexuous, boring, cockscrew and serpentine movements may be observed.

In wet preparations, leptospires cannot be seen by light-field microscopy but can easily be seen with dark-field illumination or phase-contrast microscopy. Although they are Gram negative, they appear faintly stained with aniline dyes. They can readily be stained by several silver impregnation techniques,

Congo red and fluorescent antibody technique. Leptospires are obligate aerobes that have optimum temperature of 28-30°C with a generation time of six to 16 hours. They require long chain fatty acids, vitamins B_1 and B_{12} for growth. The fatty acids are the major energy and carbon sources but the nitrogen source is the ammonium salt. Leptospires are oxidase positive and chemoorganotrophic. Carbohydrates and amino acids are not suitable sources of carbon and energy. A pyrimidine analogue, 5-fluorouracil is used in selective media for the isolation of leptospires from contaminated sources because leptospires incorporate purines but not pyrimidines into their nucleic acids. The media used for the cultivation of leptospires are those enriched with rabbit serum (Fletcher , 1928; Korthof ,1932; Stuart , 1946) or bovine serum albumin (Ellinghausen and McCullough, 1965; Johnson and Harris, 1967) or protein-free (Shenberg, 1967; Bey and Johnson, 1978). Semisolid media are useful for isolation and maintenance of leptospires. Solid media are used for cloning the strains and for isolating leptospires from contaminated sources. Bacteriophages and plasmids have not been isolated from leptospires.

Environmental conditions determine the survival rate of leptospires. Leptospires require optimum

conditions of moisture, warmth and pH values around neutrality for survival in a given environment. Dessication, excessive sunlight, pH values outside 6.2 to 8.0 range, high salinity, detergents, hypochlorites, iodine, disinfectants and high adsorptive capacity of the soil, affect the survival rate of leptospires. Leptospires are also sensitive in vitro to many antibiotics. Penicillin, streptomycin and tetracycline are useful in the treatment of leptospirosis. Officially two species of Leptospira are recognized, Leptospira interrogans representing the pathogenic leptospires and Leptospira biflexa representing the saprophytic leptospires (Johnson and Faine, 1984). A third species, Leptospira parva which was isolated from tap water and is biochemically intermediate between L. interrogans and L. biflexa, has been proposed (Hovind-Hougen et al., 1981). Also a second genus, Leptonema, in the family Leptospiraceae and with a single species, Leptonema illini, has been proposed (Hovind-Hougen, 1979).

2.3.1 Taxonomy

Leptospira interrogans consists of strains which parasitize a wide variety of mammalian hosts, usually resting in the kidney, whence cells may be shed in the urine (Faine, 1982). *Leptospira biflexa* consists of saprophytic strains which are commonly found in natural surface water and occasionally in sea water. The two species are currently arranged into serovars based on their antigenic composition. In addition to pathogenicity, *L. interrogans* differs from *L. biflexa* by its inability to grow at $13^{\circ}C$ (Johnson and Harris, 1967) and in the presence of 225µg/ml 8-azaguanine (Johnson and Rogers, 1964). Neill *et al.* (1983) observed that the level of hydrolysis of the preferred substrate for strains of *L. interrogans* was distinctively higher than that demonstrated for *L. biflexa*. Also using aminopeptidase substrate specificity profiles, Neill *et al.* (1987) differentiated pathogenic from non-pathogenic leptospires. Other methods of differentiation include the ability of the cells to convert to spherical forms by 1M NaCl (Johnson and Faine, 1984), lipase activity and 2,6-diaminopurine

sensitivity (Johnson and Harris, 1968) and guanine plus cytosine (G+C) content of the species (Johnson and Faine, 1984).

2.3.2 Classification

Leptospira was classified previously on the basis of host specificity, disease symptoms, carrier hosts and geographical distribution (Schuffner, 1938). The isolation and description of many serovars from different parts of the world have limited the above criteria for classification. Consequently many different methods have been used in the classification of Leptospira but the cross-agglutinin absorption technique (Wolff and Broom, 1954) remains an invaluable method of classification. The use of the term serotype (serological types) or serovar (serological varieties) instead of species was adopted to designate strains of the same antigenic constitution. Two strains are considered to belong to different serovars if after cross-absorption with adequate amounts of the heterologous antigen, 10 per cent or more of the homologous titre regularly remains in at least one of the two antisera in repeated tests (WHO, 1967). Serological typing of a

new isolate can be achieved first by determining the serogroup using selected antisera (group sera). The isolate is then tested with antisera to different serovars within the serogroup to narrow the number of possibly related serovars. On the basis of observed cross reactions, representative strains of serovars are chosen for reciprocal agglutinin - absorption tests for definite identification. Preliminary culture typing to serogroup level can be carried out in ordinary diagnostic laboratory and the definitive typing is usually carried out in leptospirosis reference laboratories (Faine, 1982). The serogroup serves as a guide in selecting antigens and antisera for the preliminary serological characterization of isolates. Approximately 180 different serovars arranged within 19 serogroups on the basis of cross-agglutinin absorption tests have been reported for Leptospira interrogans (Johnson and Faine, 1984).

Other classification methods have been described but some of them are too complicated for use in ordinary diagnostic laboratory while others have not gained general acceptance. Using deoxyribonucleic acid (DNA) hybridization, Haapala et al. (1969) and Brendle et al. (1974) identified six DNA relatedness groups. Between groups, strains share

partial sequences of nucleic acid. The serological relationships do not necessarily correlate with genetic relationships although serologically closely related strains appear to be genetically homologous. Genetic groups may therefore contain serologically diverse serovars. Yasuda et al. (1987) proposed seven new species of Leptospira on the basis of DNA relatedness and concluded that serogrouping did not equate with species identification, as serovars from several different serogroups belonged to different species. The (G+C) content of DNA in Leptospira interrogans is 35.3-39.9 moles per cent and the type strain is ATCC 23581 which is the reference strain for serovar icterohaemorrhagiae (Johnson and Faine, 1984).

Kmety (1967) proposed another method of classification on the basis of the antigenic structure of the serovar. Accordingly, he characterized the serovar by a specific arrangement of its "major or main" agglutinogens. Many different agglutinogens are present in leptospires forming a mosaic which is different for each serovar. One agglutinogen or a combination of agglutinogens forms a "factor". The factors of the serovars belonging to a given serogroup are assembled and tabulated to form

a scheme which shows the specific arrangement of factors for each serovar (Dikken and Kmety, 1978). The incubation of a strain with a battery of "factor sera" will reveal which factors are present, that is corresponding to those in the "factor sera" that cause agglutination. Investigation of an unknown strain by factor sera never proves the definite serovar status of the strain but gives a strong indication. Every typing by factor sera must be completed by the classical cross-agglutinin absorption test with the indicated reference strain (Dikken and Kmety, 1978). This factor analysis method of classification appears complicated and only performed by specialized laboratories.

The application of restriction endonuclease analysis (REA) to the classification of leptospires was first proposed by Marshall et al. (1981). The method has proved to be more sensitive and discriminating taxonomic tool than serotyping (Thiermann et al., (1985). Le Febvre et al. (1985) described REA as the most accurate method of proper identification of Leptospira. This technique examines and compares leptospires by their genetic makeup, rather than their surface components and it

eliminates the undesirable problem of cross-reactions characteristic of most serological classification methods.

Using a set of 13 monoclonal antibodies, Terpstra et al. (1985) classified almost all serovars within the Sejroe serogroup. Monoclonal antibodies allow a precise definition of antigens which give them an important place in the classification of microorganisms and therefore also in the quality control of culture collection. However, the polyclonal antisera have a considerable degree of cross-reactivity so that extensive and repeated cross-agglutination absorption tests are necessary for serovar classification which is based on quantitative differences (WHO, 1967).

Leptospira biflexa shares the morphological, cultural and physiological characteristics of the genus Leptospira except that strains can grow at 13°C and are usually resistant to 225µg/ml of 8-azaguanine. Some strains isolated from sea water are sodium-dependent and will not grow at sodium concentration below 0.24M. L. biflexa is serologically heterogenous as determined by agglutination and cross-agglutinin absorption tests. More than 60 different serovars arranged in 38

serogroups have been described for L. biflexa (Johnson and Faine, 1984) based on the antigenic relatedness. Genetically, three different groups of strains have been demonstrated on the basis of DNA relatedness. Between groups, strains share partial sequences of nucleic acid and no genetic homology with strains of L. interrogans has been demonstrated (Johnson and Faine, 1984). Strains of L. biflexa are not pathogenic in experimental animals commonly susceptible to Leptospira interrogans. The (G+C) content of DNA in Leptospira biflexa is 38-41 moles per cent and the type strain is ATCC 23582 which is also the reference strain for serovar patoc strain Patoc 1.

2.4 Bovine Leptospirosis

After the description of human leptospirosis, the disease was described in cattle in Russia by Mikhin and Azhinov (1935). The causative agent, Leptospira grippotyphosa, was isolated from a natural case and proved experimentally to be pathogenic for calves (Semskow, 1941). A high incidence of abortion due to leptospirosis was described in the United States of America (Matthews, 1946) after the first

report of the disease by Jungherr (1944). Nikolajev (1946) implicated L. grippotyphosa as the cause of epizootics in cattle in the U.S.S.R. and the same serovar was described simultaneously in Israel by Bernkopf (1946). Interest in bovine leptospirosis increased considerably at this time and many reports from different parts of the world ensued.

Baker and Little (1948) showed that L. pomona could cause atypical mastitis in cows and haemoglobinuria in calves. Sutherland et al. (1949) in Australia demonstrated leptospires in kidney sections from an aborted foetus. Bovine leptospirosis has been reported from almost all parts of the world and in many countries it constitutes a major problem in cattle industry rivalling brucellosis (WHO, 1959). Reinhard (1953) and Mitchell (1959) stated that L. pomona was responsible for bovine abortion in many countries. Van Riel and Van Riel (1955) in Central Africa, and Bodi et al. (1964) in Hungary reported serological evidence of bovine leptospirosis due to L. grippotyphosa. Leptospira canicola was isolated from the urine of a two-day-old calf (van der Hoeden, 1955; Turner et al.; 1958).

The symptoms most frequently encountered in bovine leptospirosis include abortion, stillbirth,

foetal death, weak calves, retained placenta, decreased milk production, mastitis and infertility (Amatredjo and Campbell, 1975; Faine, 1982). Serological surveys conducted in many parts of the world have shown that the Sejroe (Hebdomadis) serogroup is the major serogroup responsible for bovine leptospirosis (Amatredjo and Campbell, 1975). Leptospira hardjo is increasingly being recognized as the commonest cause of bovine leptospirosis (Ellis, 1984). A predominance of Sejroe (Hebdomadis) serogroup has been reported in Kenya (Ball, 1966), Senegal (Van Riel et al., 1971), Sudan (El-Vali, 1979) and Nigeria (Ezeh and Agba, 1982).

2.4.1 Epidemiology

Cattle can be infected by any one of the large number of pathogenic leptospires but only a small number of serovars will be endemic in any particular region or country (Blackmore and Schollum, 1980). Leptospirosis is a disease which shows natural nidality and each serovar tends to be maintained in specific maintenance hosts (Blackmore and Schollum, 1980). The natural hosts of pathogenic leptospires are the domestic and wild animals. In any particular

region therefore, cattle will be infected by the predominant pathogenic serovar particularly *hardjo* which has been shown to have worldwide distribution (Amatredjo and Campbell, 1975). Cattle are now recognized as the maintenance host for *hardjo* and there is no wildlife reservoir (Little *et al.*, 1980). Once introduced into a herd, *hardjo* spreads fairly rapidly and the leptospire is excreted in the urine for prolonged periods ranging from a few months to many years. Other serovars can infect cattle depending on the availability of incidental and maintenance hosts.

Investigations of wildlife in various parts of the world have revealed that leptospires are widely distributed (Turner, 1967). Pathogenic leptospires have also been isolated from wild birds, fish, reptiles and frogs (Michna, 1970; Turner, 1967) and also from laboratory rats and mice. Urine of healthy or diseased carriers may contaminate soil, pastures, water, foodstuff, beddings, utensil and serves as potential source of spread (Amatredjo and Campbell, 1975). Kidneys of carrier animals, meat from animals slaughtered during leptospiraemia and occasionally from diseased or convalescent cattle may play an important role in the epidemiology of leptospirosis

(Michna, 1970). Thus enzootic leptospirosis may exist in cattle herds and other animals with a possibility of transmission from one species to another and to man. Leptospires appear to have a special affinity for the pregnant cow and the foetus. The relative importance of incidental infections to cattle is determined by the opportunity which prevailing management and environmental factors provide for contact and transmission of leptospira between incidental hosts and cattle.

Because of environmental conditions, leptospiral infections of accidental hosts are abundant in tropical and subtropical regions and occur throughout the year (Thiermann, 1984). In temperate regions, however such infections will be seasonal that is, during the rainy and warm months (Doherty, 1967). Animals suffering from clinical infection and the convalescent animals continue to excrete leptospira in their urine, thus providing a continuous source of infection for varying length of time.

2.4.2 Transmission

Spread of pathogenic leptospirae occurs not only between cattle but probably also from other

animals or man (Amatredjo and Campbell, 1975). Transmission of leptospirosis results from either direct or indirect contact with infected urine. Direct transmission from bovine to bovine can occur through infected urine, post-abortion uterine discharge, infected placenta, sexual contact or *in vitro* infection (Ellis *et al.*, 1986). Other direct transmission include cutaneous contact with contaminated water (Doherty, 1967), transplacental (Fennestad and Borg-Petersen, 1962), venereal (Sleight and Williams, 1961) and conjunctival (Van der Hoeden, 1955). Since the transmission of serovar *hardjo* is independent of external factors like rainfall (Hoare and Claxton, 1972; Elder and Ward, 1978), and management (Hanson *et al.*, 1965; Higgins *et al.*, 1980), direct transmission is important in the epidemiology of bovine leptospirosis. Subsequent entry of pathogenic leptospires into fresh hosts may be through the skin, mucous membrane, nose or muzzle, the mouth or the conjunctiva (Turner, 1967).

Sexual modes of transmission have been suggested because leptospires have been observed in the semen of bulls (Sleight *et al.*, 1964) and in the genital tract of non-pregnant cattle (Ellis *et al.*, 1986). *Leptospira pomona* survived in frozen bovine

semen for one month at - 190°C (Jones, 1958), for one month at 3-4°C and for three years at - 196°C (Rodina, 1971). Transmission of leptospires in cattle by coitus and artificial insemination has been demonstrated (Sleight and Williams, 1961). Thiermann (1982) reported that serovar hardjo can persist in the bovine kidneys for more than 18 months and it is possible that some animals become lifelong carriers. The sucking of genitalia, scrotum, teats and ears of other animals in a herd and urine drinking may increase the spread of the disease to susceptible cattle (Sutherland, 1950). Mitchell et al. (1960) demonstrated clinical mastitis by experimentally inoculating infected milk into the udder of a healthy cow. Serovar hardjo has been recovered from milk of an experimentally infected cow from days 30 to 91 post-infection (Thiermann, 1982).

Indirect transmission is more important in the transmission of incidental infections. It occurs through exposure to an environment contaminated with infected material from cattle or incidental hosts. This method is favoured by environmental factors which permit the maximum survival of leptospira (e.g. warmth, moisture, neutral pH values) and management system which facilitates close contact between the

carrier and susceptible animals (Hanson et al., 1965). Most of these conditions are found in the tropics at all seasons and in the temperate zones only during summer and autumn (Turner, 1967). In cattle, transmission of leptospirosis by ticks is possible. A strain of grippotyphosa was isolated from the tick Dermacentor marginatus found on cattle (Krepkogerskaya and Rementsova, 1957) and L. canicola was cultured from Rhipicephalus sanguineus on a hedgehog (van der Hoeden, 1958). There is also a possibility that flies may act as vectors of leptospires which could be picked up from urine or any infected material and be deposited later on the mucosa of conjunctiva or nostril, on skin wound or contaminated foodstuff (Michna, 1970).

The prevalence of bovine leptospirosis has not been accurately determined due to the difficulties experienced in isolating many leptospires from clinical materials, lack of uniform isolation media, and lack of uniformity in the interpretation of serological results. Moreover, antigens used in serological surveys frequently may not have adequately represented the serovars present in a particular region. Arbitrary significant titres have also been used by various workers in the

interpretation of serological survey data although it is known that such arbitrary values have no merit in the investigation of cattle-adapted hardjo which induces low antibody titres (Ellis et al., 1982b). While there are numerous reports on the serological prevalence of leptospirosis in many tropical regions of the world, few bacteriological surveys have been carried out (Ellis, 1984).

2.4.3 Pathogenesis

The pathogenesis of leptospirosis is poorly understood (Amatredjo and Campbell, 1975). Infection of susceptible cattle occurs through the mucous membranes or abraded or water-softened skin and this is followed after 4-10 days incubation period by a bacteraemic phase which may last from a few hours to 7 days. This phase is characterized by pyrexia, excretion of leptospires in milk and with some serovars, by functional damage to the internal organs (Ellis, 1984). There is great variation in virulence between serovars and even between strains and the strains my be modified by environmental conditions or by prolonged residence in the host (Wilson and Miles, 1966). Parasitized animals may not be equally

susceptible to the pathogenic effects of the leptospires. Some species of rodents are known to tolerate several serovars including icterohaemorrhagiae (Amatredjo and Campbell, 1975).

Pathogenesis of leptospirosis has been attributed to cytotoxic, haemolytic, toxic and immunologic factors (Diallo, 1978). During the bacteraemic phase, leptospires will invade internal organs and the extent of the damage will depend on susceptibility of the host and virulence of the organisms. When circulating antibodies appear in the circulatory system of the animal, the leptospires localize and persist in the proximal renal tubules of the kidney and in the female genital tract (Ellis et al., 1986). Pathogenic leptospires are subsequently excreted in urine for a variable period of time depending on the infecting serovar and the age of the animal. Urinary excretion lasts only for several weeks in incidental hosts but with serovar hardjo , excretion can last for up to 542 days and possibly for the animal's lifetime (Thiermann, 1984). Urinary excretion is probably the most important factor in the transmission of leptospirosis. The initial kidney infection is characterized by oedema, haemorrhages in the glomeruli, cloudy swelling and necrosis of

proximal tubular epithelium (Hanson, 1982). Death in acute leptospirosis is primarily due to severe kidney lesions although concurrent hepatic lesions can contribute to fatal infections (Hanson, 1982). Amatredjo and Campbell (1975) reported that at all stages of infection the tissue changes do not necessarily correlate with the degree of leptospiral shedding, as in some animals large numbers of leptospires may be shed in the urine without concurrent significant lesions in the kidneys Although abortion occurs in the later half of pregnancy in cows, it is not a constant sequel to leptospirosis.

2.4.4 Clinical Signs of Bovine Leptospirosis

Clinical signs of bovine leptospirosis are frequently not observed and inapparent forms are more common (Sullivan, 1974). The acute leptospirosis is often characterized by severe septicaemia with pyrexia, anorexia, haemoglobinuria and jaundice especially in young calves (English, 1979). The mortality rate may be high and if recovery occurs, there is usually prolonged convalescence period. In lactating cows production of yellow clotted milk,

which may be blood-stained occurs early followed by agalactia which in most cases persists from a few days to 2 weeks. Although most cattle return to a normal level of lactation in two to three weeks (Sullivan and Callen, 1970), some fail to reach their full potential during the entire lactation period. In dairy herd affected by serovar hardjo there may be marked drop in milk let down. All four quarters of the udder are affected and the udder feels soft and flabby (Ellis et al., 1976). Agalactia may not be recognized easily in beef cattle but the production of weak calves is evident due to lack of adequate milk intake (Hanson, 1980).

Clinical evidence of nephritis occurs in both acute and chronic forms of leptospirosis. Fatal infections are generally due to severe renal degeneration. Infertility has often been associated with the less acute disease most frequently caused by members of the Sejroe serogroup. The most important consequences of bovine leptospirosis are abortion, retained placenta, stillbirth and premature live birth of weak calves (Thiermann, 1984). Abortion has been diagnosed at all stages from the fourth month to term and there is strong evidence that serovar hardjo may also cause embryonic death (Ellis,

1983). Chronic leptospirosis involves inflammatory and degenerative changes in the kidneys and eyes of the infected cattle. Chronic nephritis is associated with persistent kidney infection while the ocular lesions result from either persistent infections or reinfection of sensitized tissues with the same serovar (Hanson, 1977). The severity of leptospiral infection is determined by an interplay of factors and the general condition of the host (Turner, 1967). Skin lesions associated with chronic leptospirosis in cattle have been described (Gayot, 1955). Serovar pomona was recognized as the main serovar responsible for bovine abortion in many countries (Mitchell, 1959; Hanson, 1977) but today serovar hardjo is the most prevalent leptospira incriminated in bovine abortion throughout the world (Higgins et al., 1980; Ellis, 1984). Serovar hardjo is a well-adapted cattle parasite causing infection in which agglutinin levels are low or undetectable (Thiermann, 1983). The economic losses due to leptospirosis appears difficult to quantify but if only one aspect is considered, - prenatal death - it is possible to envisage the enormity of the problem. Holroyd (1980) reported that in a six year split herd vaccination experiment with a serovar hardjo bacterin conducted

in tropical northern Queensland that vaccination caused a significant ($P < 0.1$) reduction of prenatal loss (from 3.6 to 1.4%/year). If this 2.2 per cent reduction represent the losses due to hardjo in cattle in tropical countries, then in South and Central America with an estimated total cattle population of 250 million, the annual calf loss due to leptospirosis would be enormous. Steele et al. (1957) reported that leptospirosis was recognized as the fourth most important disease of cattle in the United States of America.

2.4.5 Isolation of Leptospires from Cattle

The isolation of the infecting leptospires ideally will confirm and help in the interpretation of any serologic result. Isolation of leptospires therefore can be used for the diagnosis of leptospirosis although the procedure is often difficult and frustrating (Turner, 1967). Isolation attempts for the diagnosis of bovine leptospirosis have been limited by such factors as lack of suitable isolation medium, lack of uniformity of such medium and lack of trained and experienced laboratory staff

in most laboratories Thiermann, 1984; Ellis, 1984). Where the above factors have not been fully considered and taken care of, the strains isolated must be considered as those which the methods used could isolate and not necessarily the most prevalent leptospires present. A variety of media have been used for the successful isolation of leptospires from bovine specimens. The media consist of those enriched with rabbit serum for example Korthof's medium or the Tween 80 bovine serum albumin medium of Ellinghausen and McCullough (1965) and the modification of it by Johnson and Harris (EMJH) (1967) or the low protein media. Supplements like fresh rabbit serum (1%) when added to EMJH is said to enhance isolation rate (Ellis et al., 1986). They also recommended topping up of cultures with fresh medium as a means of increasing the chances of isolating hardjo from bovine specimens. For the control of contaminating bacteria, 5-fluorouracil (100µg/ml or more) as well as extensive dilution of the sample in bovine serum albumin have been recommended (Thiermann, 1980). In selecting proper tissues for isolation, it is necessary to determine the stage of infection in the animal. In chronic infection, isolation from the midstream urine of a second voiding after the administration of furosemide is recommended (Nervig and Garrett, 1979).

2.4.6 Control of Bovine Leptospirosis

The most common method of control is vaccination with or without supplemental antibiotic therapy (dihydrostreptomycin at a dosage of 25mg/kg body weight). Work by Doherty (1967) and Stalheim (1969) has shown that single systemic dihydrostreptomycin treatment at 15-25mg/kg and 25mg/kg body weight respectively were effective in eliminating serovar pomona infection from the kidneys of experimentally - infected cattle. They attributed this result to the peculiar property of the drug to accumulate in the renal and uterine tissues. However, Ellis et al. (1985) reported that treatment of experimentally infected non-pregnant heifers with dihydrostreptomycin (25mg/kg) failed to eliminate established genital or renal hardjo infection in seven of the 10 heifers. Vaccination of cattle with a bacterin of an appropriate serovar has been shown to be effective and reliable for the prevention of bovine leptospirosis (Hanson et al. 1972). For routine vaccination in closed-herds, all cattle should be immunized annually and in open-herds revaccination every six months is advocated. Ideally, multivalent bacterins should contain the serovars enzootic in a

given country since there is rarely any cross-protection between serovars (Thiermann, 1984). This requirement according to Ellis (1984) can be a major drawback to the use of bacterins in tropical countries since the endemic serovars have not been adequately identified in many such countries. Sterility problems related to endemic bardjo herd infections usually decrease following vaccination with a specific bacterin, and vaccinated cattle develop low agglutination titres (about 1:100) even after repeated annual vaccinations (Hanson, 1980). According to Diesch (1980) a titre of even 1:100 is questionable if serum sample is obtained within 2-3 months of repeated vaccinations. He further stated that following vaccination with commercial multivalent bacterins significant titres develop but persist for only 1-3 months. Until better methods of totally preventing leptospiruria in vaccinated cattle become available, vaccination at least for the present, remains the method of choice for the control of bovine leptospirosis since the reservoir for leptospires is composed of a broadspectrum of animal species.

2.5 Diagnosis of Leptospirosis

A variety of laboratory aids are available for the diagnosis of leptospirosis in animals and man. The diagnosis is not usually difficult but the identity of the infecting strain can only be made reliably by isolating and identifying it (Turner, 1967). Based on the clinical signs and symptoms alone, the diagnosis of animal and human leptospirosis is difficult because those signs are not usually pathognomonic. Consequently diagnosis is often dependent on an evaluation of individual and herd histories, clinical signs, lesions and laboratory findings (Faine, 1982). Although culture of blood or cerebrospinal fluid can give a confirmatory diagnosis, the organisms grow slowly *in vitro*, often requiring weeks for detection and identification. The isolation of leptospires by direct culture from blood obtained from patients during leptospiraemic phase is, if properly executed, the most reliable of all procedures available for the diagnosis of human leptospirosis (Feigin and Anderson, 1975). Isolation of leptospires from blood or cerebrospinal fluid generally can be achieved only during the first 10 days of clinical illness although

isolation has occasionally been made from samples obtained as late as the 19th day of illness (Edwards and Domm, 1960). Leptospires appear in the urine during the second week of the disease and may persist for 30 days or longer. In rare cases, human leptospirosis can be diagnosed by examining tissue sections obtained by biopsy or at necropsy (Feigin and Anderson, 1975).

Leptospirosis should be considered in the differential diagnosis of any pyrexia of unknown origin (PUO) especially in patients with occupational or recreational risk of exposure (Turner, 1967). Since Leptospira is geographically a widely distributed genus evoking a wide range of clinical manifestations of varying severity which may be undiagnosed or misdiagnosed, the incidence can only be determined by investigation and no longer is it advisable to assume that it does not occur because the so-called "classical" syndromes are unknown in any particular country (Turner, 1968). Most of the available laboratory text-books have been written with the acutely ill, hospitalized, human patient in mind and not domestic animals where the chronic sequels to infection such as abortion and chronic renal carrier state pose particularly difficult

problems for the veterinary diagnostician. Although the diagnosis of leptospirosis requires few special equipment like darkground microscope, many practising physicians and veterinarians in the tropics often consider the disease too rare to include in the differential diagnosis of febrile illnesses or are ignorant of the disease. Moreover most diagnostic laboratories in the tropics are ill-equipped to make conclusive diagnosis of leptospirosis. The few reference laboratories with adequate facilities for the diagnosis of leptospirosis are finding it difficult to cope with the demands on their services (Turner, 1967). As a result, reports on isolates take undue lengthy periods before they are known.

According to Turner (1967), laboratory diagnosis of leptospirosis in both animals and man may be achieved by:

i. visualization of leptospires in urine, blood or tissues using darkfield microscopy, fluorescent antibody technique, silver impregnation technique, immunogold and immunoperoxidase techniques;

ii. demonstration of specific antibodies in the serum or tissue fluid of the animal

or patient by macroscopic or microscopic agglutination test, complement fixation test, indirect haemagglutination test, enzyme-linked immunosorbent assay;

iii. isolation of the infecting serovar by direct culture in different media or by inoculation into susceptible laboratory animals followed by subsequent isolation from their blood or kidney.

Microscopy: The examination of urine, tissue fluids, blood and tissue suspension can be used to demonstrate typical leptospire and this may aid in prompt diagnosis of lepospirosis. There are usually too many serum proteins and cell debris artifacts which may resemble intact or partially intact leptospires (Pseudospirochaetes) (Rahman and Macis, 1979) and in urine, particularly of cattle, the concentration of organisms is frequently too low for them to be recognized by this method (Ellis, 1986). A presumptive diagnosis of leptospirosis is made only if typical morphology and motility of leptospires are demonstrable and this diagnosis must be confirmed by cultural or serological methods (Sulzer and Jones, 1974). However a diagnosis of acute leptospirosis

should never be made following demonstration of leptospires in kidney only as such a finding could be coincidental even in the hands of very experienced laboratory staff (Ellis, 1986).

Silver impregnation technique: The technique depends on the ability of the method to selectively coat the surfaces of the leptospires with a deposit of silver so that they can be visualized with ordinary light microscopy. Leptospires are usually poorly stained with conventional bacteriological stains. Various modifications of Levaditi's silver impregnation technique have been successfully used for the demonstration of leptospires in tissues and body fluids especially if the number of organisms present is few or in instances where isolation attempts are frequently unsuccessful (Sulzer and Jones, 1974). This staining method cannot differentiate between infecting serovars and other tissue elements which are argentophilic. Well-stained preparations show black spirochaetes in a pale-yellow or colourless background which may be counterstained by haematoxylin and eosin (H & E) stain. The method has been used for epidemiological surveys of wildlife and for

retrospective demonstration of leptospires in fixed pathological specimens (Faine, 1982), but it is too complicated and unreliable (Terpstra et al., 1983).

Immunoperoxidase staining: Immunoperoxidase staining can be used for the rapid diagnosis by direct demonstration of leptospires in clinical specimens. Sophisticated optical equipment is not required for the examination of stained material. Cox and Twigg (1981) successfully used the technique in the histopathological examination of tissues in experimental leptospira infection. Immunoperoxidase staining can be used to differentiate between "pseudospirochaetes" and true leptospires and the materials so stained can be examined by ordinary light microscopy in semi-permanent slides (Terpstra et al., 1983). Leptospires can only be visualized when present in large numbers since the contrast between leptospires and the tissue background is usually poor. Where the number of leptospires is few, differential centrifugation to concentrate the organisms (Wolff, 1954) or examination of plasma layer just above the buffy coat in centrifuged microhaematocrit tubes (Mackintosh and Thompson, 1979) will presumably enhance staining efficiency.

Immunogold staining: Because all immunofluorescent stains fade with prolonged storage or prolonged examination and fluorescent microscope is required for the demonstration of leptospires, immunogold silver staining method was developed. Immunogold silver staining technique used in visualizing leptospires in routine paraffin sections provides a useful alternative to silver impregnation and immunofluorescence techniques in the diagnosis of leptospirosis (Skilbeck and Chappel, 1987). The leptospires are intensely stained black and clearly visible against the eosinophilic stained kidney tissue. The technique is specific because no leptospire-like structures were seen in immunogold silver stained sections of tissues which were culture - negative for leptospires (Skilbeck and Chappel, 1987).

Fluorescent antibody technique: The method was reported by White and Ristic (1959) who examined formalinized urine containing pomona but had some difficulty in obtaining serovar specificity. By this method, reactions are more pronounced with homologous or serologically closely related serovars and are less intense or negative with heterologous serovars.

The test has the advantage of detecting leptospiral antibodies as early as four days after onset of infection (Sulzer and Jones, 1974). Direct fluorescent antibody (FA) test has been used to demonstrate leptospires in urine and tissues (Cook, 1970) but the sensitivity and specificity of the various modifications of the technique have not been sufficiently standardized for routine diagnostic use (Hodges and Ekdahl, 1973; Sulzer et al., 1975). The principal advantage of the FA technique is the speed and accuracy with which the identity of a serovar can be determined provided that high titred antisera were used for preparing conjugates and that the optimal dilution of each conjugate was first determined (Hodges and Ekdahl, 1973). Dacres (1961) also reported that leptospiral serovars could be distinguished by the FA technique when osmic acid vapour was used as a fixative. The test is serogroup specific and a valuable tool for screening of tissue materials and urine. It can be used in conjunction with other serological tests for the confirmation of doubtful results. Serological tests are preferred to direct visualization of leptospires in the diagnosis of leptospirosis because of the presence of artifacts which can easily be confused with leptospires (Sulzer and Jones, 1974).

Macroscopic agglutination test: A rapid macroscopic agglutination test described by Stoenner (1954) and Galton *et al*. (1958) can be used to screen human and animal serum samples for evidence of leptospirosis. The test is used for the detection of acute and recent infections but it is considered less sensitive than the microscopic agglutination test and it is more broadly reactive. Slide test antigens are suspensions of leptospires which have been killed by formalin, thiomersal or by heating. The antigen so prepared are stable for at least one year provided that it is stored at 4°C (Turner, 1968). Galton *et al*. (1958) demonstrated a broad cross-reactivity with many different antigens within the genus when screening human and rabbit sera but with bovine sera, cross-reactivity was limited.

The Mazzonelli-Mailloux slide test (Galton *et al*., 1974) uses a heat-killed concentrated Patoc 1 antigen. The test detects leptospiral antibodies irrespective of the infecting serovar but it is unsuitable for surveys or screening for evidence of previous infection (Faine, 1982). It is however useful in detecting current infections in human sera.

Microscopic agglutination test (MAT): The microscopic agglutionation test (MAT) using live antigens is the most widely used standard reference test for the serodiagnosis of leptospirosis because of its high specificity and sensitivity (Arimitsu et al., 1982) and is the basis for other serological tests (Faine, 1982). It requires multiple serovars of live leptospira involving the risk of infection to the operator and maintenance of a large number of stock cultures to provide antigens. Equal volumes of serum and antigen are allowed to react for one to two hours after which the degree of agglutination and end - point titre are determined by examining the mixture by darkfield microscopy (Sulzer and Jones, 1974). The MAT titre is determined as the highest serum dilution showing agglutination of at least 50 per cent of the leptospires. The sensitivity of the test is improved when local isolates are included in the battery of antigens used for the MAT (Gray, 1982 ; Ellis, 1986). When dead antigens are employed the antibody titres obtained is usually less than the corresponding one obtained using live antigens (Turner, 1968). Some of the disadvantages of MAT according to Bey and Johnson (1986) include the necessity for maintenance of a large culture bank

which is potentially hazardous to the laboratory personnel, the subjectivity in the reading, the time required for the test, the inability to detect low titres in some vaccinated hosts and serovar specificity. A significant MAT titre in an unimmunized animal is 1:40 or greater for serovar hardjo and 1:100 or greater for other serovars. However in a vaccinated animal, an MAT titre of 1:40 is considered suspicious if vaccination was not given within the previous 10 days (Shotts, 1976; Stringfellow et al., 1983). There is not one single MAT titre that can be considered diagnostic for all cases but herd studies can give more useful information than single samples (Thiermann, 1984). In areas where vaccination is practised, antibodies to multivalent bacterins can interfere with serologic diagnosis of leptospirosis (Hanson, 1982) since even a titre of 1:1000 is questionable if the sample tested was obtained within 2-3 months of repeated vaccination (Diesch, 1980). The comparison of antibody titres in paired serum samples as recommended in most textbooks (Turner, 1968; Sulzer and Jones, 1974) is of little value unless the first sample was collected during the acute stage of infection (Diesch, 1980). A negative reaction does

not rule out the possibility of infection, since the patient may be infected with a serotype not included in the battery of testing antigens (Sulzer and Jones, 1974).

Complement fixation test (CFT): This test is useful in detecting current and recent past infections (Turner, 1968). The test does not appear to be very useful in testing animal sera (Ris, 1975). Hodges and Weddell (1977) described a semi-automated CFT capable of testing 288 serum samples per hour. The test is genus-specific using serovar patoc as antigen. It is less serotype-specific and uses killed antigens (Hodges and Ris, 1974). Compared with MAT, the CFT is easier and quicker to read and the antigen can be stored for longer period of time. However CFT is less sensitive than the MAT and it does not detect low levels of antibody (Ellis et al., 1982b). Another major disadvantage of CFT is that several test adjustments have to be made when the test is applied to different animal species (Turner, 1968; Shotts, 1976).

The indirect haemagglutination test (IHAT): This test was developed to avoid the difficult task of maintaining numerous leptospiral serovars in active

growth for the serodiagnosis of leptospirosis (Cirone et al., 1978). The test uses soluble antigen extracted from serovar patoc to sensitize sheep erythrocytes which are fixed with glutaraldehyde. The test is genus specific (Sulzer and Jones 1973). Compared with MAT, the IHAT is positive only with sera from persons with current leptospiral illness and the fixed cells may be stored for at least one year. From the diagnostic point of view IHAT offers no particular advantage over the MAT but when used in parallel it may be possible to distinguish recently infected cattle from those with a chronic infection (Morris et al., 1977).

Enzyme-linked immunosorbent assay (ELISA.): ELISA was reported by Azouaou et al. (1980) as a valuable aid in screening herds for the presence of antibodies to leptospira especially in areas where several serovars are known to occur. Terpstra et al. (1980) also used a genus-specific test with no specificity for antibody class and concluded that ELISA was sensitive and relatively easy to perform. Thiermann and Garret (1983) reported that the ELISA test was more sensitive than MAT, detected all shedder animals but was unable to differentiate vaccination from

infection titres. Thiermann (1983) using serum samples from vaccinated cattle detected 64.0 per cent with *hardjo* antigen using ELISA whereas the MAT could detect only 18.0 per cent positive. The ELISA uses only one heat-extracted antigen which is sufficiently broadly reactive to detect antibodies induced by different serovars. Terpstra *et al.* (1980) observed that ELISA and MAT results agreed in 95.0 per cent of the sera from 96 leptospirosis patients and concluded that ELISA as a genus-specific test, is a valuable addition to the serodiagnosis of leptospirosis. Faine (1981) stated that ELISA procedure offers promise for objective and relatively simple serological diagnosis and mass screening of man and animals. It is however obvious that serologic methods in general are of restricted value in the diagnosis of leptospirosis especially if they are not used in conjunction with isolation of infecting serovar (Turner, 1968; Thiermann, 1984).

2.5.1 Cultural Isolation

Isolation of infecting leptospiral serovar by direct culture in different media is rewarding provided that suitable media and clinical materials

are used. Leptospires can readily be isolated from the blood of patients during febrile or leptospiraemic phase of illness. Leptospires usually appear in the urine during the second week of the disease and may persist for 30 days or longer (Sulzer and Jones, 1974). Various media are commonly used for the cultivation of leptospires and they include media supplemented with rabbit serum, those in which whole serum is replaced by Tween 80 and bovine albumin (fraction V) and the low protein media (synthetic media comprising chemically defined ingredients, fatty acids or tweens). The rabbit serum has been shown to contain high concentration of vitamin B_{12} which is essential for the multiplication of leptospires and it may also possess chelating properties or possibly be able to detoxify inhibitory factors introduced with the inoculum or produced during growth of the cultures (Turner, 1970).

The media containing serum which have been used for the isolation of leptospires include Fletcher's (1928), Korthof's (1932) and Stuart's (1946). Modifications of these are still in use in various laboratories of the world. The serum from other animal species are inferior to rabbit serum (Turner, 1970). The Tween 80 bovine serum albumin medium (EM)

of Ellinghausen and McCullough (1965) and the modification of it(EMJH) by Johnson and Harris (1967) have been reported better than the serum-containing media. Ellis (1986) reported that EMJH + 1 per cent fresh rabbit serum and Tween 80/40 medium containing sodium pyruvate, lactalbumin hydrolysate, superoxide dismutase and bovine serum albumin were superior to EM and EMJH for the cultivation or isolation of Leptospira hardjo. For isolation purposes these liquid media are usually converted to semi-solid media by the addition of purified agar to a final concentration of 0.1 - 0.2 per cent. Often clinical materials are contaminated and various inhibitors in graded concentrations are added to the existing media to make them selective. Stavitsky (1945); Nahmias and Galton (1960) found that low concentrations of bacteriostatic agents have little or no effect on the growth rate of leptospires but selectively inhibited other microorganisms when incorporated in the growth media. Johnson and Rogers (1964a) suggested the use of 5-fluorouracil as a selective agent for the growth of leptospires. Adler et al., (1986) modified EMJH by adding six antibiotics to produce a superior selective medium for the isolation of hardjo and pomona serovars from clinical material. Contaminated

cultures may also be inoculated into hamster or guinea pig intraperitoneally and the animals bled after 15 minutes by heart puncture. The blood is inoculated into several semi-solid media and incubated (Sulzer and Jones, 1974). In all cases, materials to be cultured are highly diluted to increase the isolation chances of leptospires. Fungal contamination can be prevented by the incorporation of cycloheximide (actidione) at a concentration of 0.5mg/litre (Consineau and Mckiel, 1961). Laboratory animals like hamsters, guinea – pigs, gerbils (meriones), chinchillas and also day-old chicks are often used to purify contaminated cultures containing leptospires. The chicks appear to be slightly more refractory to small numbers of leptospires than weanling hamsters and guinea pigs (Sulzer and Jones, 1974).

The inoculated animals are kept for some weeks, then sacrificed and direct cultures made from leptospira predilection sites such as the kidney, heart blood and liver. Gochenour et al. (1961) argued that the use of laboratory animals for the isolation of leptospires offered no great advantage over direct culture of tissues and body fluid on synthetic media. Faine (1981) suggested that test animals may only be used in reference and research laboratories but not

for diagnostic purposes. But Alexander et al. (1962) were of the opinion that the presently used media cannot supplant entirely the use of laboratory animals for the isolation of leptospires. Although there has been tremendous improvement in the preparation of isolation media, it is known that the Syrian hamster is highly susceptible to a wide variety of pathogenic leptospires and may therefore not be easily replaced by yet any known leptospiral growth medium. In living animals the urine is the specimen usually used for the isolation of leptospires (Shotts, 1976) although animal urine is often grossly contaminated (Sulzer and Jones, 1974). The urine collected in sterile containers should be inoculated immediately into appropriate media or laboratory animal. If immediate inoculation is not possible, urine should be diluted 1:10 in sterile saline because leptospires are inactivated in undiluted urine within three hours (Turner, 1970).

The successful diagnosis of leptospirosis will depend largely on the ability of the clinician to evaluate epidemiological data and clinical findings as well as laboratory confirmation (Heath et al., 1965).

2.6 Human Leptospirosis

Leptospirosis is thought to be the most widespread zoonosis in the world (van der Hoeden, 1964) and it has been reported in most countries where deliberate effort has been made to find it. Human leptospirosis is caused by any pathogenic serovar belonging to the species, Leptospira interrogans. In the past, clinicians used different complicated nomenclature to define leptospiral infections caused by various serovars. For example, Inada et al. (1916) used "Weil's disease" to describe infection in human caused by serovar icterohaemorrhagiae, "seven-day-fever" (hebdomadis), Ito et al. 1918; "autumn fever" (autumnalis), Koshina et al., 1925; "rice-field-fever" (bataviae), Babudieri, 1938; "swamp" or "mud-fever" (grippotyphosa) Tarassoff, 1931; "swineherd's disease" (pomona), Gsell, 1944 and "Fort-Bragg fever" (autumnalis) Gochenour et al., 1952. Similarly such terms were widely used in veterinary practice to describe disease entities caused by leptospiral serovars in animals. However with better knowledge of clinical manifestations, improved laboratory diagnostic aids together with current understanding of the widespread distribution

of leptospires in many species of domestic and wild animals, it is no longer necessary to use such ambiguous nomenclature in describing human or animal leptospirosis (Michna, 1970).

In man, leptospirosis varies considerably from subclinical to acute fatal infection. Some serovars are more virulent than others and human leptospirosis is not synomymous with Weil's disease (Turner, 1967; Michna, 1970) because most leptospiral infections in man do not cause such severe illness. The natural reservoir for many pathogenic leptospires is wild animals particularly rodents (Waitkins, 1985). Domestic animals such as dogs or farm livestock may also become infected either directly or indirectly by contact with contaminated urine from another host. The domestic animals may maintain enzootic leptospirosis in their herd from whence transmission can involve other animals and man. Man is usually an accidental or "dead-end" host (Turner, 1967). The common clinical signs of human leptospirosis include fever, chills, headache, myalgia, nausea and vomiting although severe manifestations such as icterus, haemoglobinuria, nephritis and meningitis may occur (Kaufman, 1976). Since clinical manifestations of leptospirosis vary considerably, leptospirosis in the

early stages should be considered in the differential diagnosis of influenza, brucellosis, Q-fever, meningitis, poliomyelitis, rubella, hepatitis A, glomerulonephritis, urinary tract infection and toxoplasmosis (Turner, 1967; Faine, 1981).

2.6.1 Epidemiology

Torten (1979) stressed that almost all known mammals should be considered possible carriers and disseminators of leptospires within their own species as well as to other mammals and man. The epidemiology of leptospirosis is a constantly changing dynamic process in which a shift in host-parasite relation renders varying relative importance to new and old hosts (Torten, 1979). To understand the epidemiology of human leptospirosis it is necessary to determine the maintenance or reservoir host as well as accidental or incidental hosts. According to Audy (1958), a reservoir host is one which ensures the perpetuation of a particular population of parasites without the intervention of other incidental hosts. The characteristics of a maintenance host in terms of leptospiral infection have been defined by Blackmore and Hathaway(1979) as follows:

1 High susceptibility of the host to specific infection.

2. Relatively low pathogenicity of the organism for the host.

3. Long term kidney infection in relation to the systemic phase of infection.

4. Natural transmission within the host species.

They further defined a maintenance population as "a population of species of animal which acts as a continuous reservoir of a serotype in a particular ecosystem".

1. Low susceptibility to infection.

2. If the infection is established, the pathogenic effect may be severe.

3. A short renal phase.

4. Inefficient intraspecies transmission.

The rat (Rattus norvegicus) provides a maintenance population for icterohaemorrhagiae in many parts of the world. It can be infected readily with small numbers of leptospires and large numbers of leptospires may be present in the rat kidney without provoking any marked cellular response. Excretion may be lifelong and transmission readily takes place amongst rats after they have achieved sexual maturity

(Little, 1986). Turner (1967) emphasized that, in general, a particular animal species serves as a reservoir of only one specific serovar but, in addition, may be infected by other serovars, thereby acting temporarily as an incidental host for those serovars.

Man is susceptible to pathogenic leptospires but acts as an accidental host. Where infection does occur in man, it is usually serious and sometimes may be fatal. Excretion of the organism in the urine of survivors lasts for only a few weeks and man to man transmission or to other animals appears to be exceptionally rare (Feigin and Anderson, 1975; Faine, 1982). Romanian workers in North Vietnam suspected that in human vegetarians who excreted alkaline urine, man acted as a reservoir host (Spinu et al., 1963). Serovar icterohaemorrhagiae was excreted in urine of an infected human for 103 days from the onset of infection (Uhlenhuth and Fromme, 1930). Johnson (1950) recorded uninterrupted leptospiruria lasting 11 months in a man who recovered from australis B infection. Serovar poi was isolated from the urine of a girl who had recovered from leptospirosis (Borg-Petersen and Fennestad, 1956). Bernkopf et al. (1948) reported on outbreak of human

leptospirosis in Palestine which resulted from consumption of raw milk obtained from convalescent cows infected by grippotyphosa. This outbreak proved that pathogenic leptospires can survive in undiluted milk for three days. For many years in the United States of America (U.S.A.) as in other parts of the world, the rat was considered the sole animal host for icterohaemorrhagiae even though Randall and Cooper, (1944) isolated the same agent from a naturally infected dog. The earlier concept of Weil's disease being an occupational risk in sewer workers, miners, farmers, rice and sugarcane field workers, abattoir personnel, fishermen and others exposed to wet environments is no longer completely consistent with the epidemiologic features of the disease (Reif and Marshak, 1973). Also exposure by swimming in a pond or stream contaminated with urine from wild or domestic animal carriers is not a prerequisite for an outbreak of leptospirosis because almost two thirds of 472 cases diagnosed in the United States of America occurred in winter months well past the season for recreational exposure to contaminated water (Heath et al., 1965).

Blackmore (1981) reported that the annual national average of human leptospirosis in New

Zealand is 488 which is 300 times that recorded in the U.S.A. and 80 times that in the United Kingdom (U.K.). Although Leptospira pomona was previously reported the commonest cause of human leptospirosis (Kirschner et al., 1952) most human cases are now caused by Leptospira hardjo in many parts of the world (Faine, 1982). These changing patterns of leptospirosis have been reported in the U.K. where at least four to five per cent of all dairymen are at risk of cattle-associated leptospirosis (Waitkins, 1986). Also in Britain, it is estimated that approximately one third of all cattle herds are infected by Leptospira hardjo and 10 per cent of all cattle abortions are asscciated with high level of leptospiral antibody to serovar hardjo (Little et al., 1980; Ellis et al., 1982a).

2.6.2 Transmission

The transmission of leptospiral infection from an animal to man occurs directly by contact with blood, tissues, organs or urine of infected animals or indirectly by exposure to an environment contaminated by leptospires (Feigin and Anderson, 1975). Doeleman (1932) reported a case of Weil's

disease in a woman who was presumably infected by her convalescent husband at coitus. Many outbreaks of human leptospirosis have often resulted from bathing, swimming, fishing or canoeing in waters contaminated with urine of carrier farm animals (Turner, 1967). Also contaminated foodstuff, pastures, soil and premises have often been implicated in the transmission of leptospirosis in man. Handling of infected animals or their organs at or after slaughter, the mopping of urine and accidental bites by carrier rodents and dogs may result in the transmission of pathogenic leptospires from animal to man (Michna, 1970). Following direct exposure to infected animals, the acquisition of leptospiral serovars by man may take place through breaks in the skin or via the mucous membranes including conjunctivae, vagina and nasopharynx (Feigin and Anderson, 1975). Accidental laboratory infections with leptospires have been reported and infection is usually associated with puncture by contaminated needles or broken glass-ware, handling infected laboratory animal or through contamination of the conjunctivae with infective materials (Turner, 1967).

2.6.3 Pathogenesis

Invasion of the fully susceptible host results in leptospiraemia which usually lasts for four to five days, occasionally for seven days and terminates as a result of the appearance of specific antibodies and subsequent phagocytosis clearing leptospires from circulation and their localization in the kidneys (Burnstein and Baker, 1954). Clinical and histological findings in human and animal leptospirosis may in part be the result of toxins, enzymes or other metabolites elaborated by or released from lysed leptospires (Alexander et al., 1956; Faine, 1957; Imamura et al., 1957; Bauer et al., 1961). Pathogenic leptospires have been shown to produce hemolysin (Bauer et al., 1961) and it is likely that haemolysis occurs in selected cases of severe leptospirosis in man and this may contribute to the development of jaundice (Edwards and Domm, 1960). Impaired renal function is an important cause of death in many leptospirosis patients and interstitial nephritis is the fundamental renal lesion observed in leptospirosis especially in patients suffering from chronic infection (Hutchison et al., 1946). Renal failure in human leptospirosis is primarily the

result of tubular damage. In patients dying within the first week of illness, kidney changes included cloudy swelling or isolated tubular epithelial cell necrosis involving mainly the distal convoluted tubule, isolated mild interstitial oedema with sparse lymphocytic infiltration (Hutchison et al., 1946). In patients dying during the second week of illness, the renal lesions are more prominent. Numerous foci of tubular epithelial necrosis are apparent, in addition to interstitial oedema and infiltration of lymphocytes, monocytes, plasma cells and neutrophils (Feigin and Anderson, 1975). Leptospiral meningitis is, according to Gsell (1944); Edwards and Domm, (1960), an expression of an antigen-antibody reaction, since meningeal inflammation occurs only after antibody titres have had time to develop.

2.6.4 Clinical Manifestation

Clinical symptoms of human leptospirosis vary considerably depending on the infecting serovar. Leptospirosis may be described as an acute systemic infection characterized by an extensive vasculitis but a large number of subclinical infections also occur, (Feigin and Anderson, 1975). In cases of

leptospirosis which are clinically recognizable, only about five to ten per cent exhibit features of Weil's syndrome (Feigin and Anderson, 1975). The diverse and often non-specific manifestation of leptospirosis coupled with low index of suspicion of this disorder on the part of physicians undoubtedly account for the considerable number of unrecognized cases (Heath et al., 1965). About 90 per cent or more of all cases of human leptospirosis are anicteric but patients present with fever, headache, severe muscular pain, malaise, prostration and even circulatory collapse (McCrumb et al., 1957). Conjunctival suffusion, photophobia and ocular pain as well as conjunctival haemorrhage are specific and of diagnostic importance (Feigin and Anderson 1975). Human leptospirosis varies from inapparent to severe infection and even death. A rash due to subcutaneous and internal haemorrhages may be observed. The clinical signs observed may vary from only fever and inappetence to jaundice, depression, haemoglobinuria, nephritis and pneumonia (Hanson, 1982). Edwards and Domm (1960) emphasized that in all the patients suffering from leptospirosis, neutrophilia is always present regardless of the total white blood cell count. Although an incubation period of 7 to 12 days has

been reported by most investigators, the range varies from 2 to 20 days (Turner, 1973). Faine et al. (1984) reported probable human abortion due to serovar hardjo.

2.6.5 Treatment and Control of Human Leptospirosis

Although some investigators (Edwards and Domm, 1960; Turner, 1967) have advocated immediate treatment of patients prior to tissue damage, it is known that it takes a long time to establish the diagnosis of leptospirosis. It is difficult to assess the efficacy of antimicrobial agents used in the treatment of human leptospirosis since the disease is self-limiting and even severe cases of Weil's disease have often recovered without specific therapy (Beamer, 1952). Hall et al. (1951) compared the therapeutic effects of penicillin, chloramphenicol, aureomycin and terramycin with placebo in 67 confirmed cases of leptospirosis. They were unable to demonstrate any appreciable effect on the duration or severity of illness or on the prevention or amelioration of the central nervous system, hepatic, renal or haemorrhagic complications in treated patients with any of the antibiotics employed. Also

the duration of leptospiraemia and the persistence of the organism in the cerebrospinal fluid were not altered by the treatment. Consequently they concluded that antibiotics were of no value in the treatment of human leptospirosis. Similar experiment by Fairburn and Semple (1956) using chloramphenicol and penicillin in 83 confirmed patients agreed with the above results and they stated that even when these antibiotics were initiated on or before the fourth day of illness, there was no marked advantage in using the antibiotics. Wintrobe et al. (1974) stated that penicillin, streptomycin, tetracycline, chloramphenicol, erythromycin and oleandomycin are all effective even though data on their efficacy are conflicting. Turner (1967) advocated the use of penicillin in high doses and if renal function is impaired recourse to peritoneal dialysis or artificial kidney should not be delayed. He suggested that since penicillin may not eradicate the leptospires from the kidneys, supplementary administration of streptomycin would be desirable. Ellis et al. (1985) reported that the administration of dihydrostreptomycin at the recommended dose of 25mg per kilogram body weight (Stalheim, 1969) did not remove established genital or renal leptospiral

infection. Despite the large number of antimicrobial agents available to clinicians, the therapeutic efficacy of any of these drugs for the treatment of human leptospirosis remains doubtful (Feigin and Anderson, 1975).

With the widespread distribution of leptospires in wildlife and the continuous presence of asymptomatic carriers, the control of human leptospirosis seems difficult. Although leptospiral vaccines prepared in protein-free media have been tested (Shenberg and Torten, 1973; Christopher et al., 1982), they have not been widely accepted as a method of controlling human leptospirosis. Even in animals where they are widely used, the role of vaccines in preventing leptospiruria remains doubtful (Allen et al., 1982). But Flint and Liardet (1980) had claimed that a trivalent vaccine in cattle prevented urinary shedding and protection persisted for at least 12 months. Since leptospirosis in man is acquired principally from exposure to urine of infected cattle (Flint and Liardet, 1980; Reed and Liardet, 1981), an obvious control measure would be the limitation or prevention of urinary shedding by vaccination of animals (Blackmore et al., 1981). Marshall et al., (1982) tested a commercial

pomona-hardjo vaccine which had previously been shown to be effective against hardjo infections, concluded that the vaccine represented the most effective tool in the prevention of leptospirosis in cattle and those who work with them. However, Bettelheim and Fogg (1986) observed that vaccinating cattle with serovar hardjo would not necessarily reduce the human incidence of leptospirosis in dairy milkers. The vaccination of animals already shedding leptospires in their urine has no significant effect in reducing the prevalence of leptospiruria and there is no evidence to support the use of pomona-hardjo vaccine in already infected herd (Hancock et al., 1984).

The control of human leptospirosis can be achieved by strict observance of personal hygiene, environmental hygiene, prophylactic vaccination and revaccination of stocks on regular basis and treatment of acute cases with dihydrostreptomycin (Turner, 1967; Bey and Johnson, 1986). All secretions and excretions from infected animals should be considered infective and surface waters that may be contaminated by animal urine should be considered a possible source of infection (Hanson, 1982). Finally all occupational groups at risk should be educated on the simple methods of avoiding infection.

2.7 Leptospirosis in Rodents

Leptospirosis is one of the most widespread zoonoses in the world (Feigin and Anderson, 1975) and rodents represent the most important reservoirs of leptospires. Shortly after the identification of Leptospira icterohaemorrhagiae in man by Inada et al. (1916), the significance of the rodent as a vector and reservoir of icterohaemorrhagiae became apparent. Following the first isolation of Leptospira icterohaemorrhagiae in the United States of America, the rat was considered as the sole animal host for the serovar in many parts of the world. This serovar had also been isolated from other animal species including cattle, swine, goats, hamsters and dogs (Randall and Cooper, 1944). The low incidence rate of leptospirosis reported in many parts of the world may be related to the failure of clinicians and epidemiologists to recognize the more common anicteric forms of leptospirosis as well as belated recognition that animals other than rodents could transmit leptospirosis (Feign and Anderson, 1975).

The rat (Rattus norvegicus) is the predominant natural carrier of icterohaemorrhagiae in most regions of the world (Thiermann, 1981). The primary

carrier of serovar ballum is the house mouse (Mus musculus) although other rodents and small mammals are often infected (Hanson, 1982). The black rat (Rattus rattus) is the carrier of canicola and icterohaemorrhagiae (Brown and Gorman, 1960). The West African brown field rat (Arvicanthus niloticus) has been reported as a carrier of serovar icterohaemorrhagiae in Egypt (McGuire and Myers, 1957). A particular host species may serve as a reservoir for one or more serovars of leptospires and also a given serovar may be hosted by multiple animal species. Two or more reservoir hosts for the same serovar may be present in the same geographic area, and individual animals may carry and excrete strains of leptospires belonging to multiple serogroups (Babudieri, 1958; Turner, 1967).

In Britain, Field and Sellers (1950) implicated rodents as the reservoir of Leptospira icterohaemorrhagiae which was isolated from an affected calf. Clark et al. (1962) isolated grippotyphosa from voles (Microtus pennsylvanicus) trapped on the farms and from stream water while Mitchell et al. (1960) reported an outbreak of hardjo leptospirosis in cattle associated with feeding fruits which had previously been eaten by field rodents. van der Hoeden (1958) and van der

Hoeden et al. (1967) showed that the hedgehogs were carriers of L. canicola and they could play an important epidemiological role in the spread of leptospirosis to man and domestic animals. Fennestad (1956) reported an eight per cent incidence of leptospirosis in Danish cattle and suggested that the striped field mouse (Apodemus agrarius) acted as natural host of pomona. Michna and Campbell (1969) isolated L. sejroe from bovine kidneys and postulated that infection might have been acquired from pasture contaminated by urine of feral rodents, shrews or voles which proved to be reservoir hosts. There is some evidence that wild life associated with infected livestock can remain relatively free of leptospirae (Gordon-Smith et al., 1961; Doherty, 1967).

Songer et al. (1983) isolated L. ballum from three house mice (Mus musculus) and from 358 trapped rodents. In Senegal, Baylet and Van Riel (1971) isolated icterohaemorrhagiae from the kidneys of white-tailed rats (Cricetomys gambianus) and detected significant antibody titres against icterohaemorrhagiae, canicola and butembo in the rats. Ball (1966) in Kenya, isolated four strains of leptospires belonging to Icterohaemorrhagiae and Grippotyphosa serogroups from gerbils (Tatera robusta) and further in a

serological examination of 547 rodent sera only 12 were positive with significant titres to Icterohaemorrhagiae and Autumnalis serogroups. In an extensive survey of leptospirosis in 6606 rodents, Kranendonk et al. (1969) isolated 256 leptospires belonging to Icterohaemorrhagiae, Autumnalis, Hebdomadis, Tarassovi, Australis, Javanica, Canicola and Bataviae serogroups. In a bacteriological survey of leptospirosis in Zaria, Nigeria, Diallo and Dennis (1982) recovered eight isolates comprising australis, ballum and two unidentified strains from 252 trapped rodents. They also detected leptospiral antibodies to serovars australis, icterohaemorrhagiae, tarassovi and hardjo. The spectrum of serovars present in farm animals and man in a given area reflects the pattern present in the wildlife of that area (Twigg et al. 1972).

Chapter 3

SEROLOGICAL PREVALENCE OF BOVINE LEPTOSPIROSIS IN PLATEAU STATE OF NIGERIA

Summary

Serum samples obtained from 1537 cattle in the 14 local government areas (LGAs) of Plateau State of Nigeria were screened for the presence of leptospiral antibodies using 13 leptospiral serovars in a modified microscopic agglutination test (MAT). A total of 14.4 percent of these cattle had leptospiral antibody titres of 1:100 or higher to one or more of the test antigens. Antibodies to serovar hardjo was the most prevalent in the 14 local government areas but there were no reactions to serovars celledoni and shermani. The prevalence rates of antibodies to individual serovars were: hardjo (35.6%), pomona (11.7%), pyrogenes (11.7%), canicola (9.5%), grippotyphosa (7.7%), bratislava (5.9%), icterohaemorrhagiae (5.9%), ballum (4.5%), autumnalis (3.6%), bataviae (2.3%) and tarassovi (1.8%).

The serological prevalence in various local government areas differed significantly ($P<0.05:X^2$). The prevalence rates of leptospiral antibodies

detected in the 14 LGAs of Plateau State in decreasing order were Nassarawa (28%), Shendam (20.6%), Lafia (20.5%) Akwanga (17.6%), Barkin-Ladi (14.2%), Langtang (12.8%), Jos (12.7%), Mangu (11.6%), Bassa (11.5%), Keffi (10.3%), Wase (10.3%), Pankshin (10.2%), Kanam (9.1%) and Awe (7.9%). The prevalence of sero-reactors was highest in the more fertile local government areas located at the lower plains of Plateau State where cattle congregate during the dry season in search of green pasture and water.

The high reactor rates observed in this study suggest endemicity of bovine leptospirosis in Plateau State.

3.1 Introduction

Bovine leptospirosis is one of the major diseases causing significant economic losses to cattle industry throughout the world (Nervig et al., 1980). The clinical signs associated with the disease in cattle include pyrexia, icterus, anorexia, haemoglobinuria, abortion, infertility, repeat breeding, stillbirths, foetal deaths, weak calves, mastitis, "milk-drop syndrome" and death (Michna, 1970.; Ellis, 1984). Leptospirosis is caused by a

variety of different pathogenic leptospiral serovars which are morphologically indistinguishable and capable of causing similar signs and symptoms. In cattle, Pomona, Grippotyphosa, Hebdomadis, Bataviae, Autumnalis, Australis, Icterohaemorrhagiae, Canicola, Ballum, Pyrogenes and Tarassovi serogroups have been associated with clinical disease in naturally-infected cattle (Amatredjo and Campbell, 1975.; Ellis and Michna, 1976). Cattle play an important role in the epidemiology of human leptospirosis. Following the virtual eradication of Brucella abortus from cattle in many countries, the commonest cause of bovine abortion is Leptospira interrogans serovar hardjo (Leitch, 1983). In many parts of the world, the Sejroe serogroup especially serovar hardjo is being recognized as the major cause of bovine leptospirosis (Little et al., 1980.; Ellis, 1984).

In the tropical and subtropical countries of the world, literature indicates widespread serological evidence of bovine leptospirosis with few microbiological or clinical disease reports. For this reason, the antigens used in such serological surveys may not adequately represent the serovars present in a particular country or region (Ellis, 1984). The

problem is often compounded by the use of arbitrary significant titres in the interpretation of serological survey data. Although useful in eliminating some of the confusion which may arise due to cross-reactivity of certain leptospiral serogroups, the use of such arbitrary values has no value in the investigation of the cattle-adapted serovar hardjo (Ellis, et al., 1982.; Hathaway et al., 1986).

Relatively little is known of the Leptospira in African livestock (Amatredjo and Campbell, 1975). Reports of bovine leptospirosis survey in Africa are limited. But, Ball (1966) in Kenya, Van Riel et al. (1971) in Senegal, El-Vali (1979) in Sudan and Ezeh and Agba (1982) in Nigeria however found a predominance of Sejroe serogroup reaction in cattle examined. Many pathogenic serovars including hardjo, icterohaemorrhagiae, canicola, ballum, bataviae, grippotyphosa, australis, pyrogenes, tarassovi, hebdomadis and pomona have been reported by various workers in African cattle population (Holland et al., 1977; Diallo, 1978; Ezeh et al. 1987).

In Nigeria, the few serological studies carried out indicated that leptospirosis might probably be endemic and the serological prevalence of bovine

leptospirosis reported were: 48 per cent (Turner et al., 1975), 72 per cent (Holland et al., 1977), 11.9 per cent (Diallo, 1978), and 22.9 per cent (Ezeh et al., 1987). Bovine abortion, infertility and other signs suggestive of leptospirosis are usually poorly investigated in Nigeria but are generally attributed to diseases like brucellosis, campylobacteriosis, listeriosis, toxoplasmosis and where the cause is not readily ascertained, viral agents are incriminated. Leptospires are often difficult to isolate from infected cattle and clinical signs of bovine leptospirosis may not be observed (Sullivan, 1974). Consequently the diagnosis of bovine leptospirosis in most cases depends on the demonstration of specific antibodies in the blood of the affected cattle although the interpretation of the antibody levels can be difficult (Deas, 1981). The demonstration of an agglutination response in bovine serum dilutions of 1:100 or greater is indicative of exposure to leptospiral antigen and titres of 1:100 in unvaccinated animals are diagnostic (Faine, 1982).

This study was undertaken to determine the serological prevalence of bovine leptospirosis in the 14 local government areas of Plateau State of Nigeria.

3.2 Materials and Methods

3.2.1 Survey Area

Plateau State was selected for this study because it is a major refuge for cattle during the dry season when most cattle from other neighbouring states further north congregate in search of green pastures and water. A proportionately high incidence rate of human and bovine leptospirosis had previously been reported in the state (Diallo, 1978).

The Plateau highlands stand at an average height of 1,200 metres above the sea level. The landscape rises steeply from the riverine plains of River Benue in the South and descends towards Bauchi State in the North East. Although situated in the tropical zone, Plateau State is noted for its cool weather during most parts of the year. Mean temperatures of 41.5°C maximum and 18.1°C minimum have been recorded. Located almost at the centre of Nigeria, between latitudes 7° and 11°N and longitudes 7° and 25°E, Plateau State occupies an area of about 53,585sq. kilometers. The mean annual rainfall varies from 131.75cm in the southern part to 146cm on the Plateau. The rainy season extends from June to October

but the months of December, January and February are usually cold and dusty due to the dry harmattan winds. The state is administratively divided into 14 local government areas comprising Akwanga, Awe, Bassa, Barkin-Ladi, Lafia, Kanam, Jos, Shendam, Langtang, Wase, Mangu, Keffi, Nassarawa and Pankshin (Appendix 1). The main ethnic groups in Plateau State are Hausa, Berom, Eggon, Angas, Mwaghavul, Tarok, Ankwai, Kwaro, Egbira, Afo, Jarawa, Gwari, Miango, Alago, Rindre, Kanuri and Nunku (Plateau State Ministry of Information, Jos, 1980). The State is noted for its rich deposits of tin ore which in the past attracted numerous prospectors from Nigeria and abroad. Apart from the natural volcanic effects, tin prospecting further distorted the general topography of the State. Small bodies of water and man-made highlands resulting from endless excavation can still be seen in many parts of the State. These bodies of water become stagnant and provide water for man, domestic livestock and wild animals throughout the year. In some cases, they are used for irrigating dry season farms or converted into fishing ponds and swimming pools.

3.2.2 Design for the Serological Survey

An official letter was written to the state Ministry of Agriculture and Animal Resources requesting for permission and assistance to carry out the serological survey of bovine leptospirosis in the 14 local government areas of the state. After receiving the Ministry's approval, another letter giving details of the itinerary was despatched to the State's Chief Veterinary Officer and copies of the letter were sent to village heads and the ardos (Fulani chiefs), soliciting their assistance and cooperation. The veterinary field personnels were specifically requested to educate the herd owners on the importance of the survey to achieve full participation. Our team was made up of a fulani guide officially assigned to us by the Chief Veterinary Officer, a veterinary doctor, a research scientist, two laboratory assistants and an official driver. Owing to the rugged nature of the roads and the fact that the fulbe herdsmen virtually live in the bush with their cattle, a landrover vehicle was provided for the survey. The survey period covered January, 1984 to February, 1985 and sample collection proceeded from the lowlands and terminated in the

northern local government areas. Samples were collected from nomadic as well as from semi-nomadic herds.

Materials assembled for the exercise included a cooler with iceblocks, sterile bijou bottles for serum storage, vacutainer tubes with adaptors and needles, sterile 10ml syringes and needles, sterile Pasteur pipettes and rubber teats, cotton — wool, methylated spirit, sterile normal saline, beakers, mats and camping equipment, potable water, torch light and dry food packs.

The itinerary was so scheduled that each trip lasted for only five days in order to send all the batch samples collected to Vom for proper storage at -20°C and to replenish requirements. The number of cattle bled in each herd depended on the herd size and the cooperation of the herd owner. But the target was to collect blood from 10 per cent of all the herds sampled (Cole et al., 1979). Traditionally the Fulbe cattle owners are reluctant to have their cattle bled and the survey period coincided with a serious outbreak of rinderpest which drastically reduced the cattle population in Nigeria. That meant stiff opposition to sample collection from the surviving cattle by the Fulbe cattle owners. However,

when the team went out together with the anti-rinderpest campaign team, samples were collected very easily.

3.2.3 Blood Collection

Approximately 10ml of blood was collected from either the caudal or jugular vein of each animal after swabbing the bleeding site with methylated spirit. The route of collection depended largely on the degree of cooperation by the herd owner in handling the animals. Usually the animals were tied in twos, leg-to-leg, and the cattle attendants identified individual animals by names irrespective of the herd size. The vacutainer tubes were appropriately labelled with the animal's name and the laboratory number for easy traceback. Collection of blood was allowed early in the morning before the animals went out for grazing or in the evenings when the animals returned from grazing. After clotting, serum was separated with sterile Pasteur pipette. Hemolyzed samples were discarded and their number noted. All the serum samples in properly-labelled bijou bottles were temporarily stored in the veterinary out-post

refrigerator and where such facilities were lacking we obtained assistance from a nearby hospital or maternity clinic.

3.2.4 Screening Antigens

Thirteen leptospiral serovars kindly provided by Dr. W. A. Ellis of Veterinary Research laboratories, Belfast, Northern Ireland, Professor Emil Kmety of Komensky University, Czechoslovakia and Dr. S.A. Waitkins of Leptospirosis Reference Unit, Hereford, England, were used in the battery of antigens for the survey. The serovars were:

Serovar	Strain
hardjo	Hardjoprajitno
pomona	Pomona
canicola	Hond Utrecht IV
grippotyphosa	Moskva V
pyrogenes	Salinem
autumnalis	Akiyami
bratislava	Jez Bratislava
ballum	S102
bataviae	van Tienen

icterohaemorrhagiae	RGA
tarassovi	Mitis Johnson
celledoni	Celledoni
shermani	LT 821.

These antigens were selected in accordance with the recommended list for detecting infections by an unknown serovar (Faine, 1982) and also based on serovars previously reported in Africa. All the serovars were maintained in a semi-solid medium of Ellinghausen and McCullough as modified by Johnson and Harris (1967). However the antigens were examined every two months and subcultured into fresh semi-solid medium containing 5-fluorouracil at a final concentration of 150-200µg/ml (Johnson and Rogers, 1964). All the antigens used for the microscopic agglutination test (MAT) were grown in liquid EMJH medium supplemented with 5 percent sterile rabbit serum. The antigens were grown for 5-7 days at 30°C in 1/2oz screw-capped bottles and the growth was assessed using the Corning nephelometer (Corning Ltd., Halstead, Essex, England). The antigens used for the MAT were subcultured weekly to maintain them at the logarithmic phase of growth. A nephelometric reading of 25 units corresponding to

2×10^8 leptospires per millilitre (WHO, 1967) was used for the microscopic agglutination test. Antigens that were too dense were diluted with sterile phosphate buffered saline (PBS) and those too light were concentrated by centrifugation and resuspended with PBS in appropriate concentration. Antigens which appeared clumped were passed through 0.8µ millipore filter (Millipore, Buc-France) to remove small clumps of tangled leptospires which might interfere with the reading of the test.

3.2.5 Microscopic Agglutination Test (MAT)

All the serum samples were tested by the microscopic agglutination test (Cole et al., 1973) in disposable microtitre plastic plate first at a dilution of 1:100 against 4 pools comprising the 13 live antigens. All sera with titre to pooled antigens were retested in serial two-fold dilution system beginning at 1:100 through 1:3200 against each antigen within the positively reacting pool. Beginning from the highest dilution, 0.025ml of the serum dilution was transferred to a clean microtitre plate using plastic pipette droppers calibrated to deliver the same volume when held vertically. An

equal volume of filtered antigen was added to all the wells containing appropriate serum dilutions. Known negative and positive bovine sera diluted 1:10 were included in each test batch as controls. After shaking the plates to mix, they were stacked and the top plates covered with a plastic lid and incubated at 30°C for two hours before examination. The tests were read by transferring drops of the reaction mixture onto a clean glass slide using multiple microtitre diluters and examining under darkfield microscopy with low power objective. The degree of agglutination was scored as 4+, 3+, 2+, 1+ or negative depending on whether 100%, 75%, 50%, 25% or no leptospires were agglutinated respectively. The end-point or titre was taken as the highest dilution of the test serum at which 50 per cent or more of the leptospires agglutinated. Agglutination titres equal to or more than 1:100 were considered positive (Thiermann, 1983). Contaminated and clumped cultures as determined with darkfield microscopy were not used in the test. If titres to more than one antigen (multiple reaction) occurred in the same sample, all titres were recorded as contributing to the serological prevalences against different antigens.

3.3 Results

A total of 1537 cattle from 203 herds ("Rugas") were examined for leptospiral antibodies at a minimum titre level of 1:100. Tables 3.1 summarizes the distribution of major serological reactors to different leptospiral serovars in cattle in the 14 local government areas of Plateau State. Antibodies to serovars celledoni and shermani were not detected in any of the bovine sera tested. The overall prevalence rate of bovine leptospirosis was 14.4 per cent with hardjo (35.6% of the positive sera) as the predominant serovar detected serologically. The prevalence of antibodies to other individual serovars were: pomona (11.7%), pyrogenes (11.7%) canicola (9.5%), grippotyphosa (7.7%), bratislava (5.9%), icterohaemorrhagiae (5.9%), ballum (4.5%), autumnalis (3.6%), bataviae (2.3%), tarassovi (1.8%), celledoni (0%) shermani (0%).

The serological prevalence in various local government areas differed significantly ($P<0.05:X^2$). The prevalence rates of leptospiral antibodies detected in the 14 local government areas of Plateau State in decreasing order were: Nassarawa (28%),

Shendam (20.6%), Lafia (20.5%), Akwanga (17.6%), Barkin-Ladi (14.2%), Langtang (12.8%), Jos (12.7%), Mangu (11.6%), Bassa (11.5%), Keffi (10.3%), Wase (10.3%), Pankshin (10.2%), Kanam (9.1%) and Awe (7.9%). Only 9 multiple reactions were observed (Table 3.2) and the highest antibody titre of 1:1600 occurred with hardjo and canicola serovars. The median MAT titre was 1:200. Leptospiral antibodies due to autumnalis, bataviae and tarassovi were less frequently detected and in low titres.

Table 3.1 Distribution of Major Serological Reactors to Different Leptospiral Serovars in Cattle in the 14 Local Government Areas of Plateau State.

Local Govt Area (L.G.A.)	Number Tested	Number Positive	Percent Positive	Number of sera positive for Leptospira serovars*												
				hard.	pom.	pyr.	ball.	can.	aut.	brat.	grip.	bat.	tar.	cell.	ict.	sher
NASSARAWA	118	33	28.0	12	6	6	1	2	0	1	2	2	0	0	1	0
SHENDAM	102	21	20.6	7	3	2	1	2	1	1	2	0	1	0	1	0
LAFIA	127	26	20.5	10	5	4	1	2	0	1	0	1	0	0	2	0
AKWANGA	108	19	17.6	6	2	2	0	1	1	4	2	0	0	0	1	
BARKIN-LADI	106	15	14.2	8	0	3	0	2	1	0	1	0	0	0	0	0
LANGTANG	78	10	12.8	5	1	1	0	1	0	1	0	0	0	0	1	0
JOS	236	30	12.7	7	3	4	2	2	2	2	2	1	1	0	3	0
MANGU	95	11	11.6	4	1	0	1	0	1	0	1	0	1	0	1	0
BASSA	131	15	11.5	4	1	2	1	4	0	1	1	0	0	0	1	0
WASE	87	9	10.3	4	1	0	0	0	1	1	2	0	0	0	0	0
KEFFI	78	8	10.3	2	1	1	1	0	1	0	1	0	1	0	0	0
PANKSHIN	118	12	10.2	5	2	1	1	1	0	0	1	0	0	0	1	0
KANAM	77	7	9.1	3	0	0	1	1	0	0	2	0	0	0	0	0
AWE	76	6	7.9	2	0	0	0	2	0	0	0	1	0	0	1	0
TOTAL	1,537	222	14.4	79	26	26	10	21	8	13	17	5	4	0	13	0

*hardjo, pomona, pyrogenes, ballum, canicola, autumnalis, bratislava, grippotyphosa, bataviae, tarassovi, celledoni, icterohaemorrhagiae, shermani.

Table 3.2 Distribution of Antibody Titres to Leptospiral Serovars in 222 Positive Bovine Sera from 14 Local Government Areas of Plateau State.

Leptospira serovar	Number positive	Percent positive	Microscopic agglutination test (MAT) titre					
			1:100	1:200	1:400	1:800	1:1,600	1:3,200
hardjo	79	35.6	33(36)	19	15	10	2	0
pomona	26	11.7	12(14)	7	4	3	0	0
pyrogenes	26	11.7	9	11(12)	4	2	0	0
canicola	21	9.5	8(11)	5	6	0	2	0
grippotyphosa	17	7.7	7	7	3	0	0	0
bratislava	13	5.9	4	6	2	1	0	0
icterohaemorrhagiae	13	5.9	4	4	4	1	0	0
ballum	10	4.5	4	3	1	2	0	0
autumnalis	8	3.6	5	2	1	0	0	0
bataviae	5	2.3	2	3	0	0	0	0
tarassovi	4	1.8	2	1	1	0	0	0
celledoni	0	0.0	0	0	0	0	0	0
shermani	0	0.0	0	0	0	0	0	0
Total	222	14.4	90(98)	68(69)	41	19	4	0

Multiple reactions ( ) = 9

3.4 Discussion

Microscopic agglutination test was adopted because it is the standard reference test for the demonstration of leptospiral antibodies (Abdussalam et al., 1972., Cole et al., 1979). Many workers had used arbitrary significant titre of 1:100 (Higgins and Cayouette, 1978; Cole et al., 1979; International Zoo-Sanitary Code, 1982; Songer et al., 1983; Egan and O'reilly, 1985), but not one single titre can be considered diagnositic (Thiermann, 1984). In addition to a titre of 1:100 used in this study, other significant titres previously employed in the interpretation of serological reactions included 1:30 (Turner, 1968); 1:20 (Diallo, 1978); 1:200 (Little and Baker, 1950); 1:400 (Hill, 1979). Morter (1980) stated that a titre of 1:100 is no longer used as a criterion for leptospiral diagnosis because MA titres greater than 1:100 are produced by a single injection of current bacterin and these titres may persist for five to six months. The use of arbitrary significant titre has no merit in the investigation of the cattle -adapted serovar hardjo since the presence or absence of detectable serum antibodies was an unreliable guide to carrier status (Ellis et al., 1986).

However, since cattle vaccination against leptospirosis is not yet practised in Nigeria, a titre of 1:100 detected in apparently healthy cattle in Plateau State was considered significant.

The high prevalence of leptospiral antibodies in Lafia, Shendam, Akwanga and Nassarawa was expected because cattle from the upper Plateau usually congregate in these lower plains during the dry season in search of food and water. However the low prevalence rate of 7.9per cent observed in Awe local government area is difficult to explain since the L.G.A is equally situated in the lower plains of Plateau State. While Diallo (1978) detected no antibodies to hardjo in Plateau State, possibly due to fewer bovine samples of 87 he examined, this serovar was the most prevalent, accounting for more than a third of the total reactors. Another possible explanation to this trend might be due to the changing pattern in the epidemiology of bovine leptospirosis in Plateau State. Some years back, individuals and government establishments in Nigeria imported numerous cattle from various countries of the world to upgrade the indigenous stock without screening such animals for leptospirosis. It is possible that serovar hardjo, which was not

serologically detected in 1978, might have been among other exotic leptospires imported under agrarian revolution policies of both the Federal and State governments. Cattle from North America were thought to be responsible for the introduction of hardjo into Hungary (Kemenes, 1975).

Leptospira hardjo titres were reported present in more than 80 per cent of bovine herds in New Zealand (Hellstrom, 1978) and more than one third are affected in Britain (Hathaway, 1981). In the United States of America, serovar hardjo is the most prevalent leptospire affecting cattle (Nervig et al., 1980; Thiermann, 1983). Unlike other serovars which have wildlife and rodents as their principal reservoirs, hardjo has not been isolated from rodents (Ellis et al., 1981). However, Myers et al. (1977) reported the isolation of serovar hardjo from an armadillo which inhabits the grassland used for raising cattle in Argentina. Although cattle are the sole maintenance host for hardjo, the serovar has been isolated from sheep in New Zealand (Bahaman et al. 1980) and Northern Ireland (Ellis et al., 1983). Serological investigations have revealed high prevalences of hardjo titres in sheep in some countries with within-herd prevalences ranging from

49 per cent to 100 per cent (Hathaway et al., 1982). The epidemiological significance of these findings has not been determined.

The low prevalence of reactions to autumnalis, bataviae and tarassovi obtained in this study cannot possibly be due to cross-reactions such as the paradoxical reactions which are known to occur between various serogroups (Shotts, 1976) especially at low titres. Equally the high prevalence of pomona, canicola and grippotyphosa recorded is not clear because none of the serovars has ever been isolated in Nigeria.

A serological survey for leptospiral antibodies is more meaningful if all the serotypes known to occur in an area are used as antigens (Spradbrow, 1964). Using a laboratory strain of hardjo as MAT antigen, Bahaman et al. (1984) obtained a prevalence of 53 per cent but with a local hardjo field strain, a slightly higher prevalence of 58 per cent was obtained. Similarly, Ellis et al. (1982) observed that a local isolate of hardjo (Ab 56) consistently reacted at a higher titre than the commonly used World Health Organization reference strain (Hardjoprajitno) in the microscopic agglutination test. Since the prevalent leptospiral serovars in

Nigeria have not been fully identified, it is possible that the battery of antigens used in the present study was inadequate.

The prevalence rate of 14.4 per cent of bovine leptospirosis recorded in the present study is lower than 48·0per cent reported by Turner et al. (1975) and 72 per cent reported by Holland et al. (1977). The disparity in the prevalence may be attributed to the significant titre of 1:30 used by these workers in interpreting serological reaction. But Diallo (1978) used a titre of 1:20 as significant titre and reported a lower prevalence rate of 11.9 per cent in parts of Nigeria.

It is highly probable that leptospirosis is widespread in cattle population in Plateau State. Further studies aimed at isolating the prevalent serovars in Nigeria as well as identifying the maintenance and reservoir hosts should be intensified so that a national control policy can be formulated.

Chapter 4

BACTERIOLOGICAL EXAMINATION OF BOVINE AND RODENT KIDNEYS FOR LEPTOSPIRES

Summary

A total of 525 bovine kidneys obtained from the Jos Municipal abattoir were culturally examined for the presence of pathogenic leptospires. Six leptospiral strains belonging to two different serogroups were isolated. Five of the isolates were serologically homologous and belong to a new serovar in the Pyrogenes serogroup. The remaining isolate was identified as serovar hardjo (genotype hardjobovis). A seventh isolate was lost through contamination of the culture.

In a similar kidney examination of 502 rodents comprising 435 brown field rats (Arvicanthus niloticus), 23 white tailed rats (Cricetomys gambianus) and 44 house mice (Mus musculus), no leptospiral isolation was made. But serological examination of 105 serum samples obtained from live brown field rats showed that 44.8 per cent had leptospiral antibody titre of 1:100 and above to one or more of the following serovars: bratislava, pyrogenes, grippotyphosa, pomona, canicola, ballum and icterohaemorrhagiae.

4.1 Introduction

Leptospirosis is a major zoonotic disease which affects all domestic animals, wildlife and man. Bovine leptospirosis causes enormous economic loss from abortion, stillbirth, decreased milk production and infertility (Amatredjo and Campbell, 1975). Theoretically, any of the pathogenic leptospires may infect any animal species, but in practice only a small number of serovars will be endemic in any particular region or country and each serovar tends to be maintained in specific maintenance hosts (Blackmore and Schollum, 1980).

Although serological tests are useful for the diagnosis of leptospirosis (Hodges and Ris, 1974; Sullivan, 1974), they are not satisfactory for determining the identity of the infecting serovar. Cultural isolation is a better index of infection rate in a cattle herd, but it is difficult to do and often unsuccessful (Thiermann, 1983). Moreover, such isolations are often limited by bacterial contamination, lack of suitable isolation media and lack of suitably experienced laboratory staff (Ellis, 1984). One of the sequels to infection of susceptible cattle is that leptospires localize in the kidneys

and are excreted intermittently in urine (Ellis et al., 1981). Infected bovine urine is the major source of leptospirosis to other cattle and wildlife because of the volume of urine excreted and the duration of leptospiruria. Since pathogenic leptospires localize in the kidney during the leptospiruric phase, they are frequently isolated from the urine of live animals or from the kidney tissue of slaughtered animals. However, urine is usually contaminated with other microorganisms which will interfere with the isolation of leptospires (Turner, 1970; Orr and Little, 1979).

The major reservoirs of parasitic leptospires are thought to be rodents and domestic animals and the rodents may be life-long shedders (Ellis et al., 1981). Reported serological surveys have repeatedly shown high prevalence of leptospirosis in Africa but few deliberate attempts have been made to isolate the prevalent serovars from wildlife and domestic animals. In a bacteriological survey of leptospirosis in Zaria, Nigeria, Diallo and Dennis (1982) examined 252 rodent and 74 bovine kidneys and isolated 13 strains of leptospires. No other epidemiological studies had been undertaken since then as a follow-up even though previous studies had shown a high

percentage of seroreactors in man and livestock in different parts of Nigeria (Turner et al., 1975; Holland et al. 1977; Diallo, 1978; Ezeh and Agba, 1982).

The aim of this study was to attempt isolation of pathogenic leptospires from bovine and rodent kidneys.

4.2 Materials and Methods

4.2.1 Bovine Kidney Samples

Letters were written to the Jos Municipal abattoir project manager and the State Chief Veterinary Officer informing them about the proposed survey. The project manager was specifically requested to educate all the abattoir workers on the importance of the exercise. A meeting was held at the abattoir with all the sectional heads to facilitate easy implementation of the objective. In order to further obtain the workers' cooperation, they were all screened for non-specific urethritis and intestinal parasites. Mid-stream urine samples were subjected to culture and sensitivity tests and stool samples were examined microscopically. Results of this preliminary investigation were given to the project manager who

arranged for appropriate and prompt treatment of all the affected workers in the hospital. All the workers therefore promised to cooperate with the team in the collection of bovine kidney samples. Since slaughtering of the animals was done on bare floor haphazardly, it was not practicable to collect matched serum samples. If the butcher or the cattle owner refused to cooperate, the whole kidney was usually bought at market price. The surface of such kidneys was seared with heated spatula, and approximately one gram of tissue comprising the cortex and the medulla, was collected. The kidney blocks were placed into 10ml bottles containing one per cent sterile bovine serum albumin (BSA) and taken to the laboratory where they were processed within two hours of collection.

Processing of Bovine Kidneys:

The kidney samples were macerated before placing them into 10ml syringe barrel and expressed into 10ml liquid BSA. The homogenate was allowed to stand on the bench for ten minutes before three serial dilutions of 1:50, 1:500 and 1:5,000, were again made in liquid BSA. From each dilution, 0.1ml was inoculated into

three 10ml tubes of semi-solid modified Ellinghausen and McCullough medium (EMJH, Difco) (Johnson and Harris , 1967) containing 150µg/ml of 5-fluorouracil (Hoffman-LeRoche Inc. N.J.) and two per cent sterile rabbit serum. All the tubes were incubated at 30°C for 12 weeks (Hathaway et al., 1982) and examined weekly by darkfield microscopy for the characteristic leptospiral growth. Suspect tubes were subcultured into fresh enriched EMJH medium and reincubated at 30°C. Heavily contaminated cultures were discarded. After the 12 weeks period, all tubes showing no evidence of leptospires were discarded as negative. When the subcultured leptospires had grown sufficiently, they were further purified by filtering through 0.22µ millipore (Galton et al., 1962) into fresh semi-solid EMJH medium and re-incubated at 30°C. The isolates were adapted to liquid EMJH medium by several subcultures until growth was satisfactory for use as antigen. Duplicate pure cultures in semi-solid EMJH were sent to Leptospirosis Reference Laboratory, Komensky University, Bratislava, Czechoslovakia and also to the Leptospirosis research laboratory, Belfast, Northern Ireland for identification and confirmation of the isolate.

4.2.2 Rodent Kidney Samples

Rat hunters were employed to trap or kill rodents and submit their catch to the laboratory within two hours of collection for payment. Samples which were not properly collected were rejected.

Screening of rodent sera for leptospiral agglutinins: Blood samples were also obtained from all the 105 live rodents submitted and these were screened for leptospiral antibodies by the microscopic agglutination test (Cole et al., 1973) using 13 live antigens at an initial dilution of 1:100. The live rodents were first properly anaesthetized, bled by heart puncture before sacrificing.

Processing of the rodent kidneys: The abdomen of the rodent was swabbed with methyl alcohol and a median incision was made with a sterile scapel blade after securing the rodent on a dissecting tray. The intestine and the stomach were carefully reflected to expose the two kidneys. The kidneys were aseptically plucked with sterile forceps and after decapsulation, they were processed as described for bovine kidneys.

4.3 Results

Six (1.1%) leptospiral strains belonging to two different serogroups were isolated from 525 bovine kidneys obtained from Jos Municipal abattoir in Plateau State. Five of the isolates were serologically homologous. They were serotyped as a new serovar in the Pyrogenes serogroup and the sixth isolate vv1JA was serotyped as serovar hardjo by cross-agglutinin absorption test and factor analysis (Courtesy of Professor Emil Kmety who did the typing of the isolates at the Leptospirosis Reference Laboratory, Komensky University, Bratislava, Czechoslovakia).

A total of 502 rodents comprising 435 brown field rats (Arvicanthus niloticus), 23 white-tailed rats (Cricetomys gambianus) and 44 house mice (Mus musculus) were trapped in Vom, Plateau State and examined for the presence of leptospires. All the rodents were kidney culture - negative but serological examination of 105 serum samples obtained from live brown field rats showed that 47 (44.8%) had antibody titres of 1:100 or higher to one or more of the 13 leptospiral serovars used as antigens (Table 4.1). The most frequently detected serovars were

bratislava 19(40.4%), *pyrogenes* 8(17.0%), *grippotyphosa* 6(12.8%), *pomona* 5(10.6%), *canicola* 3(6.4%), *ballum* 2(4.3%) and *icterohaemorrhagiae* 2(4.3%). Serum titres ranging from 1:400 to 1:1,600 were detected in 17(16.2%) of the rats.

Table 4.1 Distribution of Leptospiral antibody Titres in Positive Sera of 47 Rodents Trapped in Vom, Plateau State.

Leptospiral serovar	Number positive	Percent positive	Microscopic agglutination test titre					
			1:100	1:200	1:400	1:800	1:1600	1:3200
bratislava	19	40.4	7	3	2	1	4	0
pyrogenes	8	17.0	1	4	3	0	0	0
grippotyphosa	6	12.8	1	0	1	4	0	0
pomona	5	10.6	3	2	0	0	0	0
canicola	3	6.4	0	3	0	0	0	0
icterohaemorrhagiae	2	4.3	0	2	0	0	0	0
ballum	2	4.3	0	0	2	0	0	0
tarassovi	1	2.1	1	0	0	0	0	0
hardjo	1	2.1	1	0	0	0	0	0
bataviae	0	0.0	0	0	0	0	0	0
autumnalis	0	0.0	0	0	0	0	0	0
celledoni	0	0.0	0	0	0	0	0	0
shermani	0	0.0	0	0	0	0	0	0
Total	47	44.8	14	14	8	5	4	0

4.4 Discussion

The isolation of serovar hardjo (genotype hardjobovis) from bovine kidney is apparently the first such report in Nigeria. Also the isolation of a new serovar in the Pyrogenes serogroup clearly indicates that until extensive surveys of wildlife and domestic animals are undertaken nationwide, the prevalent leptospiral serovars in Nigeria must remain speculative. The isolation of hardjo and its presence in cattle, man and rodents have serious implication to cattle industry and is of public health significance in Nigeria. Leptospira interrogans serovar hardjo is endemic in cattle populations of many countries (Amatredjo and Campbell, 1975; Ellis, 1984) and it has been associated with abortion (Hathaway and Little, 1983) and agalactiae (Higgins et al., 1980). Serovar hardjo can persist in the bovine kidney for more than 18 months and it is possible that some animals can become life-long renal carriers (Thiermann, 1982). Cattle are the maintenance host for serovar hardjo (Orr and Little, 1979, Ellis, 1984) which has been described as a well-adapted cattle parasite (Thiermann, 1982).

In Great Britain 30 to 50 per cent of all cattle herds are affected by serovar hardjo (Little et al., 1980) and in New Zealand, 90 per cent of the herds are affected (Hellstrom, 1978). Serological titres due to hardjo are often low, and infections regularly occur in the absence of agglutinin titres (Cole et al., 1973, Ellis et al., 1982). Consequently the diagnosis of infected herd using the microscopic agglutination is often difficult. In Africa, reactions due to serovar hardjo have been described in Kenya, Senegal, Sudan and Nigeria (Ball, 1966, Van Riel et al., 1971, El-Vali, 1979, Ezeh and Agba, 1982, Ezeh et al., 1987). In a clinical study of leptospirosis in Kwale district, Kenya, de Geus et al., (1977) isolated 9 strains of leptospires most of which belonged to the Hebdomadis serogroup. Based on the high antibody titres detected in trapped rodents (Arvicanthus niloticus), Diallo and Dennis (1982) predicted that serovars tarassovi and hardjo would someday be isolated from animal species in Nigeria. The predominance of hardjo antibody titres in cattle (Chapter 3) and its isolation in the present study confirm the existence of this cattle-adapted serovar in Nigeria.

The isolation of only six strains according to Ellis (1984) must be considered as those which the

methods used could isolate and not necessarily the most prevalent serovars present in Plateau State. Serovar hardjo is usually difficult to isolate, but with improvement in isolation media and technique (Ellis, 1986), the serovar is increasingly being isolated in different parts of the world. Ellis (1986) also demonstrated the superiority of Tween 80/40 medium containing sodium pyruvate, lactalbumin hydrolysate, superoxide dismutase and bovine serum albumin over other media used for the isolation of fastidious serovars like hardjo. In a bacteriological examination of 180 bovine organs, he reported that the recovery rate of hardjo when the original culture tube was topped-up with fresh medium and subcultured two or three times was 47.2 per cent compared with 8.3 per cent when the original culture tube was not manipulated. In the present study only tubes showing evidence of leptospiral growth were subcultured. This may have accounted for the low recovery rate of the serovar hardjo from the bovine or rodent kidney in the present study.

Diallo (1978) isolated serovar pyrogenes from bovine kidneys and the isolation of a totally different serovar in the Pyrogenes serogroup appears to be second such report in Africa. The implication

of the close relatedness of the five strains isolated on different sampling period (January, 1984 to February, 1985) is yet unknown. But it can be speculated that the slaughtered cattle originated from the same geographical area of Plateau State. Serovar pyrogenes has been isolated from water buffaloes in the south east Asia and in Peru (Diallo and Dennis, 1982).

When Hathaway et al. (1982) isolated no leptospire from 272 house mice (Mus musculus) in the southeast England, they attributed their failure to the geographical location of Britain (an island) which had resulted in a house mouse population that was genetically distinct from those in other geographic regions in terms of susceptibility to leptospiral infection. Although this explanation cannot strictly apply in the present study, Jos Plateau is situated in an ecological zone quite different from Zaria which lies in the Northern Guinea savanna zone. Ellis et al. (1981) recovered no leptospires nor detected antibodies to serovar hardjo in 143 mice trapped on leptospire-infected property and concluded that rodents were not reservoir hosts for serovar hardjo. Rats trapped in parts of Israel were free of leptospirosis but when they spread out

in search of food in leptospire-infested area, subsequent trapped rats yielded leptospires (Lindenbaum and Eylan, 1982). Songer et al. (1983) isolated three leptospires from 328 rodent kidneys and blamed the low isolation rate on poor culture media although Myers and Jelambi (1975) reported that the same medium (EM) (Ellinghausen and McCullough, 1965) was suitable for isolation of leptospira from naturally infected animals. In the present study modified EM medium supplemented with two per cent sterile rabbit serum was used for culturing both the bovine and rodent kidneys. There was no possible explanation for the apparent high serological prevalence due to serovar bratislava (18.1%) detected in this study.

Since leptospirosis in rodents and other wild mammals occasionally has been linked directly to the disease in man and domestic animals (Thiermann and Frank , 1980, Mackintosh et al., 1980), a sustained search for the prevalent serovars in domestic and wild animals in Nigeria deserves further serious consideration.

Chapter 5

SEROLOGICAL AND CULTURAL STUDIES OF HUMAN LEPTOSPIROSIS IN PLATEAU STATE

Summary

In a serological examination of 710 serum samples collected from human volunteers in the 14 local government areas of Plateau State, 128(18.0%) had leptospiral antibody titre of 1:100 and above. The prevalence of antibodies to individual serovars were: hardjo 28(21.9%), pomona 18(14.1%), canicola 17(13.3%), grippotyphosa 15(11.7%), pyrogenes 13(10.2%), icterohaemorrhagiae 12(9.4%) and autumnalis 8(6.3%). Of the 128 seropositive individuals, serovar hardjo was most common with a prevalence rate of 21.9 per cent. There was no statistical difference in the prevalence rate of leptospirosis in the different local government areas ($P > 0.05; x^2$).

Among the occupational groups examined, the abattoir workers were particularly at risk with a prevalence rate of 29.5 per cent. Also a leptospiral variant of strain Hardjoprajitno was isolated from the midstream urine of an abattoir worker . The isolation of Leptospira interrogans serovar hardjo in

man is the first such report in Nigeria. Leptospiral agglutinins were detected in 39.1 per cent of the patients suffering from pyrexia of unknown origin (PUO). The significance of high serological prevalence of human leptospirosis in relation to low isolation rate of pathogenic leptospires in Plateau State remains unknown.

5.1 Introduction

Human leptospirosis is an acute, febrile, septicaemic disease attributable to any one of a large number of serologically distinct members of the spirochaetal species Leptospira interrogans (ICSB, 1974). Man is susceptible to all the pathogenic leptospires and acts as an accidental host. Transmission of leptospiral infection from animal to man may occur either directly by contact with blood, tissues, organs or urine of infected animals or indirectly by exposure to an environment contaminated with leptospires. Although theoretically possible, transmission from person to person rarely occurs (Feigin and Anderson, 1975). The human infection varies greatly in severity and clinical manifestations depending on the dose and the

virulence of the infecting serovar, the host susceptibility and the organ systems predominantly involved (Faine, 1982). The signs may vary from only fever and inappetence to jaundice, depression, haemoglobinuria, nephritis and pneumonia. Owing to lack of pathognomonic signs in human leptospirosis, serologic tests alone are not relied upon to establish a diagnosis (Hanson, 1982). Diagnosis is therefore based on a correlation of serologic testing with clinical signs.

Human leptospirosis is a reportable zoonosis in many developed countries of the world. It is a well recognized occupational hazard for dairy farmers in New Zealand (Blackmore, 1981) and in Great Britain, a major occupational risk for farmers and those engaged in water-associated sports (Waitkins, 1985). In Trinidad, Everard et al. (1985) reported high prevalence of leptospiral antibodies among sugarcane workers. In Africa, human leptospirosis has been reported in Kenya where de Geus et al. (1969) described the first proven cases and in South Africa, Gauthier et al. (1969) isolated icterohaemorrhagiae from the blood of three patients. In Zimbabwe, Wiles (1979) reported a proven case of leptospirosis while in Congo, Van Riel

and Van Riel (1960) isolated several leptospiral strains many of which are now reference serovars (eg mwogolo, ndambari, ndahambukuje, kamituga, butembo, kambale and kisuba. In Senegal, Payet et al. (1966) diagnosed four cases of leptospirosis serologically and in Algeria, Benhamou et al. (1955) diagnosed canicola infection in a 30-year-old man. In Ghana, Rothstein (1964) on different occasions, examined 218 and 180 serum samples from infectious hepatitis patients and reported 55.6 per cent and 22.8 per cent positive respectively. Also Kinebuchi and Afoakwa (1973) reported 21 per cent positives in 99 sera obtained from suspected cases of leptospirosis.

In Nigeria, Leach and Canon (1943) reported a case of leptospirosis in southern Nigeria from a yellow fever patient. Collard (1962) demonstrated canicola antibodies in 4.5 per cent of people examined in Katsina province. Diallo (1978) noted 11.1 per cent incidence rate of human leptospirosis in the eight northern states of Nigeria and also reported the highest incidence (31.8%) of the disease in Plateau State.

This study was undertaken to determine the serological prevalence of leptospirosis in different occupational groups in the 14 local government areas of Plateau State of Nigeria.

5.2 Materials and Methods

The survey area, the screening antigens for MAT, the equipment, personnel and the organization of the human leptospirosis survey in Plateau State were basically as described in Chapter three with the following modifications:

(i) only 5ml of blood was collected to encourage other intending volunteer donors;

(ii) disposable syringes and 21-gauge needles were used for bleeding instead of vacutainers;

(iii) at the time of collecting blood samples, the donors were asked questions relating to their occupation, number of years on the same occupation, age, state of health, degree of contact with pet animals, livestock and rodents, frequency of febrile illness or headache using a questionaire (Appendix 3);

(iv) an approval letter to conduct the survey in the 14 local government areas was granted by the Chief Medical Officer of Plateau State and a copy of this approval

letter with details of the itinerary were sent to all the government hospitals, maternity clinics, dispensaries, private hospitals and village heads stressing the importance of the survey;

(v) blood was collected from all the 112 abattoir workers (18 butchers, 47 attendants, 44 casual workers and 3 meat inspectors) at the Jos Municipal abattoir. Mid-stream urine aseptically collected from all the serologically positive abattoir workers were inoculated into serum-enriched EMJH medium (Hathaway et al., 1981). All the inoculated tubes were incubated at 30°C for up to 16 weeks and examained by darkfield microscopy at two weeks intervals. Suspect tubes were subcultured or topped-up with fresh medium (Ellis et al. 1986)

A leptospiral strain isolated from one of the urine samples was tested with a battery of 13 group rabbit antisera using the microscopic agglutination test. The positively reacting sera were further titrated with the isolate and the homologous strain to determine the serogroup to which the isolate belonged.

5.3 Results

A total of 710 serum samples obtained from 598 volunteers and 112 abattoir workers in Plateau State was examined for leptospiral agglutinins. The overall serological prevalence of human leptospirosis in the 14 local government areas of Plateau State was 128 (18%) (Table 5.1). There was a statistical difference in the prevalence rate of leptospirosis in the different occupational groups ($P<0.05:X^2$). The serological prevalence rate in the different local government areas ranged from 8.1 per cent in Barkin-Ladi to 27.6 per cent in Lafia (Table 5.2). The predominant serovars detected were hardjo 28(21.9%), pomona 18(14.1%), canicola 17(13.3%), grippotyphosa 15(11.7%), pyrogenes 13(10.3%), icterohaemorrhagiae 12(9.4%) and autumnalis 8(6.3%). Other serovars were less frequently detected and there were no reaction to serovars shermani and celledoni (Table 5.3).

Of the 337 farmers examined, the overall prevalence rate was 14.2 per cent and the most prevalent serovars to which agglutinins were detected included hardjo (20.8%), grippotyphosa (16.7%), canicola (16.7%), bratislava (10.4%) and pomona (10.4%)

(Table 5.4). In the hospitalized patients, there was significant difference between groups of patients presenting different symptoms ($P<0.05; X^2$), and the pyrexic patients had the highest prevalence (Table 5.5). The predominant serovars detected in the hospitalized patients were hardjo (15.6%), canicola (15.6%), icterohaemorrhagiae (12.5%), pyrogenes (12.5%), pomona (12.5%), grippotyphosa (9.4%) and bratislava (9.4%) (Table 5.6). Among the hospital staff examined, leptospiral agglutinins were most frequently detected in attendants and cleaners (18.2%) and nurses (15.2%) (Table 5.7). Individual serovars were less frequently detected in hospital staff than in other occupational groups (Table 5.8).

Of the abattoir workers screened, 33(29.5%) were serologically positive at a titre of 1:100 and above (Table 5.9). Leptospiral agglutinins were detected in abattoir workers engaged in different operations: in meat inspectors (66.7%), in butchers (38.9%), in abattoir attendants (27.7%) and in casual workers (25%). The most frequently encountered serovars in decreasing frequency were hardjo 7(21.2%), pyrogenes 6(18.2%), icterohaemorrhagiae 6(18.2%), pomona 4(12.1%), canicola 4(12.1%) and grippotyphosa 2(6.1%) (Table 5.10).

One of the 33 mid-stream urine samples cultured was positive having yielded a leptospiral strain which reacted with serovar hardjo homologous antiserum at a titre of 1:10,000.

Table 5.1: Prevalence of leptospiral antibodies in the sera of different occupational groups sampled from 14 local government areas of Plateau State

Occupational group	Number tested	Number positive[a]	Percent positive
Abattoir workers	112	33	29.5
Hospitalized patients	126	31	24.6
Farmers	337	48	14.2
Hospital staff	110	15	13.6
Others[b]	25	1	4.0
Total	710	128	18.0

[a]Using 1:100 as cut-off titre

[b]No definite occupation

Table 5.2 Distribution of serological reactors to different leptospiral serovars in human volunteers in the 14 local government areas of Plateau State.

Local Govt. Area (L.G.A.)	Number tested	Number positive	Per cent positive	Leptospiral serovars*												
				hard.	pom.	can.	grip.	pyr.	aut.	brat.	ball.	bat.	ict.	tar.	cell.	sher.
LAFIA	58	16	27.6	5	2	1	2	2	1	0	1	0	2	0	0	0
BASSA	35	8	22.9	2	0	2	1	0	1	1	0	0	1	0	0	0
KEFFI	56	11	19.6	3	1	3	0	1	2	0	1	0	0	0	0	0
AWE	43	7	16.3	1	2	0	1	1	0	1	0	1	0	0	0	0
WASE	38	6	15.8	0	1	2	1	0	0	0	0	1	1	0	0	0
AKWANGA	51	8	15.7	4	1	0	2	0	0	1	0	0	0	0	0	0
NASSARAWA	41	6	14.6	0	2	0	1	1	1	0	0	0	1	0	0	0
KANAM	35	5	14.3	0	2	2	0	0	0	0	1	0	0	0	0	0
LANGTANG	36	5	13.9	0	2	1	0	0	1	1	0	0	0	0	0	0
JOS	56	7	12.5	2	1	1	1	1	0	0	0	0	1	0	0	0
MANGU	40	5	12.5	1	0	1	1	1	0	0	1	0	0	0	0	0
SHENDAM	34	4	11.8	1	0	0	2	0	1	0	0	0	0	0	0	0
PANKSHIN	38	4	10.5	2	0	1	0	0	0	0	0	0	0	1	0	0
BARKIN-LADI	37	3	8.1	0	1	0	1	0	0	0	0	1	0	0	0	0
TOTAL	598	95	15.9	21	15	14	13	7	7	4	4	3	6	1	0	0

* hardjo, pomona, canicola, tarassovi, australis, autumnalis, bratislava, ballum, bataviae, icterohaemorrhagiae, tarassovi, pyrogenes, shermani.

Table 5:3 Prevalence rate and antibody titres in 128 seropositive individuals for leptospirosis in the 14 local government areas of Plateau State

Leptospira serovar	Number positive	Percent positive	Microscopic agglutination test (MAT) Titre					
			1:100	1:200	1:400	1:800	1:1,600	1:3200
hardjo	28	21.9	12	10	6	0	0	0
pomona	18	14.1	7	7	2	1	1	0
canicola	17	13.3	8	5	2	2	0	0
grippotyphosa	15	11.7	7	5	2	1	0	0
pyrogenes	13	10.2	3	3	2	4	1	0
icterohae-morrhagiae	12	9.4	6	2	1	1	1	1
autumnalis	8	6.3	2	2	2	1	1	0
ballum	6	4.7	2	1	1	2	0	0
bratislava	5	3.9	0	1	2	2	0	0
bataviae	4	3.1	2	1	1	0	0	0
tarassovi	2	1.6	0	1	0	1	0	0
celledoni	0	0.0	0	0	0	0	0	0
shermani	0	0.0	0	0	0	0	0	0

Table 5.4 Prevalence rate and antibody titres in 48 seropositive farmers in Plateau State

Leptospira serovar	Number positive	Percent positive	Microscopic agglutination test (MAT) titre					
			1:100	1:200	1:400	1:800	1:1,600	1:3200
hardjo	10	20.8	4(5)	3	3	0	0	0
canicola	8	16.7	4(6)	3	0	0	1	0
grippotyphosa	8	16.7	3	4	1	0	0	0
pomona	5	10.4	2(3)	2	0	0	1	0
bratislava	5	10.4	1	2	1	1	0	0
icterohaemorrhagiae	3	6.3	2	0	0	1	0	0
pyrogenes	2	4.2	0	1	1	0	0	0
autumnalis	2	4.2	1	1	0	0	0	0
ballum	2	4.2	0	1	0	1	0	0
bataviae	2	4.2	1	0	1	0	0	0
tarassovi	1	2.1	0	1	0	0	0	0
celledoni	0	0.0	0	0	0	0	0	0
shermani	0	0.0	0	0	0	0	0	0
Total	48	14.2	18(22)	18	7	3	2	0

Multiple reaction ( ) = 4

Table 5.5 Prevalence of leptospiral seroreactors in 126 hospitalized patients presenting different symptoms in Plateau State

Patients with symptoms	Number tested	Number positive	Per cent positive
Pyrexia of unknown origin (PUO)	64	25	39.1
Muscular pain and general body weakness	23	4	17.4
Diarrhoea, stomach pain and vomiting	15	2	13.3
Severe headache and catarrh	14	1	7.1
Others[a]	10	0	0.0
Total	126	32	25.4

[a]Nervous symptoms, heart burn, progressive weight loss and inappetance

Table 5.6 Prevalence rate and antibody titres in 32 seropositive hospitalized patients in Plateau State

Leptospira serovar	Number positive	Percent positive	Microscopic agglutination test (MAT) titre					
			1:100	1:200	1:400	1:800	1:1,600	1:3200
hardjo	5	15.6	2	2	1	0	0	0
canicola	5	15.6	2(4)	2	1	0	0	0
pomona	4	12.5	1	1(2)	1	0	1	0
pyrogenes	4	12.5	2	1	1	0	0	0
icterohaemorrhagiae	4	12.5	0	2(3)	1	1	0	0
grippotyphosa	3	9.4	1	1	0	1	0	0
bratislava	3	9.4	0	2	1	0	0	0
autumnalis	1	3.1	1	0	0	0	0	0
ballum	1	3.1	0	1	0	0	0	0
bataviae	1	3.1	1	0	0	0	0	0
tarassovi	1	3.1	0	0	1	0	0	0
celledoni	0	0.0	0	0	0	0	0	0
shermani	0	0.0	0	0	0	0	0	0
Total	32	25.4	10(12)	12(14)	7	2	1	0

Multiple reaction ( ) = 4

Table 5.7 Prevalence of leptospiral seroreactors in different categories of hospital staff in Plateau State

Category of hospital staff	Number tested	Number positive	Per cent positive
Attendants and cleaners	22	4	18.2
Nurses[a]	46	7	15.2
Midwives	34	4	11.8
Doctors	8	0	0.0
Total	110	15	13.6

[a]Include student nurses

Table 5.8 Prevalence rate and antibody titres in 15 seropositive hospital staff in Plateau State.

Leptospira serovar	Number positive	Per cent positive	Microscopic agglutination test titre					
			1:100	1:200	1:400	1:800	1:1600	1:3,200
hardjo	2	13.3	1	1	0	0	0	0
pomona	2	13.3	0	1(2)	0	1	0	0
canicola	2	13.3	0	1(3)	0	1	0	0
grippotyphosa	2	13.3	1(2)	0	1	0	0	0
pyrogenes	2	13.3	0	1	0	1	0	0
autumnalis	1	6.7	0	1	0	0	0	0
bratislava	1	6.7	1(2)	0	0	0	0	0
ballum	1	6.7	0	1	0	0	0	0
icterohaemorrhagiae	1	6.7	0	0	1	0	0	0
tarassovi	1	6.7	1	0	0	0	0	0
bataviae	0	0.0	0	0	0	0	0	0
celledoni	0	0.0	0	0	0	0	0	0
shermani	0	0.0	0	0	0	0	0	0
Total	15	13.5	4(6)	6(9)	2	3	0	0

Multiple reaction ( ) = 5

Table 5.9 Prevalence of leptospiral antibodies in the sera of abattoir workers on different operations

Submission group	Number tested	Number positive	Per cent positive
Meat inspectors	3	2	66.7
Butchers	18	7	38.9
Abattoir attendants	47	13	27.7
Casual workers	44	11	25.0
Total	112	33	29.5

Table 5.10 Prevalence rate and antibody titres in 33 seropositive abattoir workers Plateau State

Leptospira serovar	Number positive	Per cent positive	Microscopic agglutination test titre					
			1:100	1:200	1:400	1:800	1:1600	1:3,200
hardjo	7	21.2	3(5)	2(3)	2	0	0	0
pyrogenes	6	18.2	3	1	2(3)	0	0	0
icterohaemorrhagiae	6	18.2	2(3)	1	0	2	0	1
pomona	4	12.1	1	2(3)	0	1	0	0
canicola	4	12.1	2	1	1	0	0	0
grippotyphosa	2	6.1	2(3)	0	0	0	0	0
autumnalis	1	3.0	1	0	0	0	0	0
bratislava	1	3.0	0	1	0	0	0	0
ballum	1	3.0	0	0	1	0	0	0
bataviae	1	3.0	0	0	1	0	0	0
tarassovi	0	0.0	0	0	0	0	0	0
celledoni	0	0.0	0	0	0	0	0	0
shermani	0	0.0	0	0	0	0	0	0
Total	33	29.5	14(18)	8(10)	7(8)	3	0	1

Multiple reaction ( ) = 7

5.4 Discussion

The serological prevalence rate of 18 per cent detected in this study is relatively high when it is considered that the serum samples were tested at an initial dilution of 1:100. Using a lower initial serum dilution of 1:20 in a similar serological survey of the eight northern states of Nigeria, Diallo (1978) reported an overall serological incidence rate of 11.1 per cent. The prevalence of leptospiral antibodies in abattoir workers and farmers was high and this result is consistent with reports from other parts of the world. In a serological survey in New Zealand, 34 per cent of dairy farmers, 23 per cent of pig farmers, 10 per cent of meat inspectors, six per cent of meat workers and five per cent of veterinarians had antibodies against leptospires (Mackintosh et al., 1980; Blackmore, 1981). In Ghana, Hogerzeil et al. (1986) identified bush farmers and cocoa plantation labourers as high risk occupational groups. When the high risk population (abattoir workers) was separated from the total volunteers tested, the serological prevalence rate was 15.9 per cent.

Although serovar biflexa has been recommended for inclusion in the battery of screening antigens for human leptospirosis serological surveys (Turner, 1968), it was not used in the present study because it gives too many cross-reactions which are difficult to interpret (Bettelheim and Fogg, 1986; Hoqerzeil et al., 1986). There was no significant difference ($P>0.05; X^2$) in the prevalence rate of human leptospirosis in the different local government areas. This result is in agreement with those of Blackmore and Schollum (1982b) that there were no significant differences between the serological prevalences of workers in different geographical regions of Waikato, New Zealand. They stated that geographical differences in serological prevalences were due to the bias created by obtaining sera from volunteers, many of whom would have had a special interest in their serological status.

Leptospira hardjo was the most prevalent serovar to which agglutinins were detected in the present study. This serovar accounted for 80 per cent of leptospirosis in cowmen and farmers in Great Britain (Waitkins, 1985) and in New Zealand, hardjo infection is responsible for two-thirds of all human leptospirosis (Blackmore, 1981). This cattle-adapted

serovar has been implicated as a probable cause of human abortion (Faine et al., 1984). The public health implication of this is obvious since hardjo is the predominant serovar infecting cattle in most parts of the world (Ellis, 1984). The serological prevalence rate of 14.2 per cent detected in farmers was lower than the 45 per cent reported among sugarcane workers in the rural community of Trinidad (Everard et al., 1985). This result was unexpected because in Nigeria, agricultural practices are still predominantly manual and conditions favourable for the propagation and transmission of pathogenic leptospires exist. Nigerian rural dwellers like in most other developing countries live in close proximity with pets and domestic animals.

In the hospitalized patients, leptospiral agglutinins were most prevalent in pyrexic patients (39.1%) and it is possible that some of those patients were suffering from active infection since leptospirosis is not routinely considered in the differential diagnosis of febrile illnesses in Nigeria. Many physicians are not aware that leptospirosis is not synonymous with Weil's disease because most human infections do not cause such severe illness (Turner, 1967) and anicteric form of

leptospirosis accounts for about 90 per cent of reported cases (Feigin and Anderson, 1975). Although none of the medical doctors examined was serologically positive at a titre of 1:100, there was no statistically significant difference in the prevalence rate of leptospirosis in the different categories of hospital staff. The attendants, the cleaners and the nurses were more likely to be infected because of their constant contact with infected patients and contaminated materials.

In Jos municipal abattoir, carcasses were carried on the head and shoulders in leaking containers and minor wounds or cuts sustained during normal abattoir operation constituted no major concern to the injured worker. It is known that leptospires usually enter the body through cuts and abrasions of the skin or by contact with mucous membrane of the nose, mouth or eyes (Michna, 1970). Abattoir workers with open wounds or cuts are therefore particularly at risk of contracting leptospirosis. In New Zealand, Blackmore and Schollum (1982a) observed the highest prevalence of leptospiral agglutinins in workers on the slaughter floor and in the present study, the highest prevalence rate of 38.9 per cent was observed in the butchers.

Serovars pyrogenes and icterohaemorrhagiae were the second most prevalent in the present study. Five leptospiral strains belonging to Pyrogenes serogroup were isolated from bovine kidneys (Chapter 4). The serovar, pyrogenes had previously been isolated from bovine kidneys in Nigeria (Diallo and Dennis, 1982). The isolation of strains in the Pyrogenes serogroup in the present study partly explains the high serological prevalence observed. The isolation of a hardjo strain from the mid-stream urine of a butcher is significant because of its role in the epidemiology of bovine and human leptospirosis in many parts of the world. Since man is considered an accidental host, leptospirosis in abattoir workers can be controlled by reducing the infection rate in beef cattle and other food animals routinely slaughtered at abattoirs and by reducing exposure to infection at the abattoir through personal hygiene.

Chapter 6

CHARACTERIZATION OF LEPTOSPIRES ISOLATED FROM CATTLE AND MAN IN PLATEAU STATE, NIGERIA

Summary

Six leptospiral strains isolated from bovine kidneys and one other strain isolated from mid-stream urine of a butcher were serologically and biologically characterized. All the bovine isolates shared serological and biological characteristics with Leptospira interrogans but the human isolate exhibited the characteristics of both pathogenic and saprophytic leptospires. All the isolates were demonstrable in fluorescent antibody-stained kidney sections of experimentally inoculated hamsters but the human isolate was not re-isolated on culture whereas other six bovine isolates were recovered culturally. The histopathological lesions in the hamster liver and kidney sections were not pathognomonic. Cross-agglutinin absorption studies indicated that five of the bovine isolates belonged to a new serovar in the Pyrogenes serogroup and the remaining two isolates belonged to serovar hardjo. Further tests using the restriction endonuclease analysis confirmed that the restriction patterns of

the five bovine isolates were identical but differed from other serovars within the Pyrogenes serogroup. The sixth bovine isolate, vv1JA, and the human isolate, vv7JH, were identified as genotypes hardjobovis and hardjoprajitno respectively. The human isolate elicited no agglutinins in inoculated hamsters but a titre of 1:20 was demonstrated in sera of hamsters inoculated with vv1JA on days 14 and 21 post – inoculation. Since serovar hardjo is increasingly being recognized as the commonest cause of bovine and human leptospirosis in many parts of the world, the human isolate, vv7JH, needs further investigation due to its perculiar characteristics.

6.1 Introduction

The pathogenic leptospires, Leptospira interrogans and the saprophytic leptospires, Leptospira biflexa are morphologically indistinguishable (Johnson and Faine, 1984). Pathogenic strains can however be differentiated from saprophytic strains on the basis of their sensitivity to 8-azaguanine (Johnson and Rogers, 1964), viability and growth at 13°C (Johnson and Harris, 1967), conversion of cells to spherical forms in 1M sodium chloride

Johnson and Faine, 1984), sensitivity to copper sulphate (Fuzi and Csoka, 1960), normal serum sensitivity (Cinco and Banfi, 1983) and pathogenicity for mammals (Johnson and Faine, 1984). Some of these tests are not widely used because they sometimes give variable results. Apart from the classical serological methods of cross-agglutinin absorption test (Kmety et al., 1970; Dikken and Kmety, 1978) and factor analysis (Kmety, 1967), the restriction endonuclease analysis (REA) of chromosomal DNA has proved to be an accurate and reliable method of comparing and classifying pathogenic leptospires (Marshall et al., 1981; Robinson et al., 1982). Detailed characterization of leptospiral isolates from a particular country or region is therefore necessary for the selection of representative screening antigens for use in epidemiological studies and research.

Reports of leptospiral isolation from animals and man in Nigeria are limited. Diallo (1978) isolated 13 leptospiral strains from rodent and bovine kidneys but identified seven of the isolates to serovar level. He identified five Leptospira australis, one L. ballum from eight rodent isolates and one L. pyrogenes from five bovine isolates. The

unidentified strains were sent to the Leptospirosis Reference Laboratory, Colindale for further identification. In the past, diagnosis of leptospirosis in man and animals in Nigeria were based on serological and microscopical evidence (Leach and Canon, 1943; Collard, 1962; Holland et al., 1977). In earlier chapters (Four and Five), seven leptospiral isolates obtained from cattle and man in Plateau State of Nigeria were sent to the Leptospirosis Reference Laboraratory and were tested using cross-agglutinin absorption test and factor analysis (Courtesy of Professor Emil Kmety of Komensky University, Czechoslovakia).

The present study was undertaken to further characterize the isolates by some serological and biological tests used in the identification of newly isolated leptospires. This work was done at the Leptospirosis laboratory, Belfast, Northern Ireland under the supervision of Dr. W.A. Ellis and his staff.

6.2 Materials and Methods

6.2.1 Source of Isolates

Six of the leptospiral strains, vv1JA, vv2JA, vv3JA, vv4JA, vv5JA and vv8JA were isolated from the kidneys of cattle slaughtered at Jos municipal abattoir. The seventh strain, vv7JH was isolated from the mid-stream urine of a butcher who was also serologically positive for leptospirosis.

6.2.2 Agglutination and Cross-agglutinin Absorption Tests

The seven isolates were purified and grown in semi-solid and liquid EMJH medium. When sufficient growth was achieved in the liquid EMJH the isolates were tested in the microscopic agglutination test (MAT) using selected rabbit group sera. Based on the MAT result, hyperimmune sera were prepared in rabbits using vv3JA representing five isolates which reacted at high titre with the Pyrogenes serogroup and vv1JA representing vv1JA and vv7JH which also reacted at high titre with Sejroe serogroup. All the isolates and the reference serovars of the Pyrogenes and Sejroe serogroups were tested against the hyperimmune

antisera using a modification of microscopic agglutination test (Cole et al., 1973). Cross agglutinin absorption tests were carried out with all reference strains which had some degree of serological relatedness to the isolates. Specific antisera against vv3JA and vv1JA were absorbed with reference serovars kwale and hardjo respectively. Again, the absorbed antisera were tested using the MAT in a doubling dilution series beginning with an initial serum dilution of 1:100.

6.2.3 Pathogenicity Studies

Groups of two 21-day-old hamsters were inoculated intraperitoneally with 0.5ml of each isolate grown in serum-enriched EMJH (Johnson and Harris, 1967) semi-solid medium. Two other groups of hamsters were similarly inoculated with Leptospira pomona and Leptospira biflexa serving as positive and negative controls respectively. Cultures used as inocula contained approximately 2×10^8 leptospires/ml. Blood was obtained at days 0, 7, 14 and 21 post-inoculation from all the hamsters and tested for leptospiral agglutinins using homologous antigens by the modified MAT. The hamsters were

observed daily for clinical manifestation of leptospirosis and sacrificed 21 days post-inoculation (Turner, 1970). The kidney and the liver were aseptically removed and examined for gross pathological lesions. After removing the capsules, the kidneys were processed and cultured as previously described (Chapter 4.2). All the inoculated tubes were incubated at 30°C for 16 weeks and observed daily for evidence of growth and those showing growth were examined by darkfield microscopy.

6.2.4 Histopathological Studies

Blocks of kidney and liver tissues from the inoculated hamsters were fixed in 10 per cent formol saline for histopathological studies. Smears and cryostat sections of kidney and liver tissues were stained by haematoxylin and eosin (H and E) method (Herr *et al.*, 1982). The kidney sections and smears were also stained with fluorescein isothiocyanate (FITC) conjugated *pyrogenes* and *hardjo* antisera (Cook, 1970; Hodges and Ekdahl, 1973; Ellis and Michna, 1976) and examined as described by Ellis *et al.* (1982).

6.2.5 Biological Characteristics

Leptospira pomona and Leptospira biflexa were included in the tests as positive and negative controls respectively.

Sensitivity to 8-azaquanine: Well grown cultures of the isolates and the controls were used in inoculating serum-enriched EMJH containing 8-azaguanine at a concentration of 225µg/ml. Inoculum used contained approximately 2 x 10^6 leptospires/ml in each case and all the inoculated tubes were incubated at 30°C for seven days. Growth was measured with Coleman Unigalvo type 20 nephelometer as described by Johnson and Gary (1962).

Viability and growth at 13°C: Cultures of the isolates containing 2 x 10^7 leptospires/ml were used in inoculating serum-enriched EMJH medium. The inoculated tubes were incubated at 13°C for 10 days. Viability and growth were assessed nephelometrically and the readings were converted to number of organisms per millilitre (Johnson and Harris, 1967).

Effect of 1M NaCl: Active and viable cultures of the isolates containing approximately 1 x 10^2 leptospires/ml were inoculated into 1M NaCl and incubated at 30°C for 2 hours. All the cultures were

examined under darkfield microscopy for the effect of sodium chloride on the isolates and the controls (Johnson and Faine, 1984).

Resistance to normal fresh serum: The isolates were inoculated into duplicate tubes containing normal fresh and heat-inactivated sera and incubated at 30°C for 30 minutes. Serum sensitivity was measured as a percentage survival of colony forming units (c.f.u.) compared with the number of colony forming units recovered in the presence of heat-inactivated serum (Cinco and Banfi, 1983).

6.2.6 Restriction Endonuclease Analysis (REA)

The protocols for the isolation and characterization of leptospiral chromosomal DNA of the bovine and human isolates studied were those of Marshall et al. (1981) as modified by Le Febvre et al. (1985). After the isolation of the DNA from the leptospires, it was digested with EcoRI digesting enzyme. Digested fragments were fractionated on 0.7 per cent agarose gel stained with ethidium bromide and photographed through a Kodak 23A filter with U.V. irradiation.

6.3 Results

The MAT results of the bovine and human isolates are summarized in Tables 6.1 and 6.2. Five of the bovine isolates reacted at high titres with serovars hamptoni and kwale. The sixth bovine isolate (vv1JA) and the human isolate (vv7JH) also reacted at high titres with serovars hardjo and sejroe. After cross-absorption with kwale and hardjo, all the five bovine strains reacted only with vv3JA (Table 6.3) while vv1JA and vv7JH reacted with reference serovar hardjo (Table 6.4).

No clinical manifestations were observed in any of the inoculated hamsters. The microscopic agglutination test (MAT) results of the inoculated hamsters are summarized in Table 6.5. Specific antibody titres detected ranged from 0 to 1:100 and there was no serological response in hamsters inoculated with Leptospira biflexa and the human isolate, vv7JH. The antibody titre (1:20) elicited by a bovine isolate, vv1JA at days 14 and 21 post-inoculation remained unchanged. No gross pathological lesions were observed in the liver and the kidney of killed hamsters. Leptospires were re-isolated from the kidney cultures of inoculated

hamsters except those inoculated with L. biflexa and the human isolate, vv7JH.

Few histopathological lesions were observed in all the kidney and liver sections stained with H and E stain (Plates 1-IV). Moderate congestion and aggregation of mononuclear cells around the tubules and blood vessels were however present (Plates 1 and II). Also moderate and diffuse degenerative changes characterized by cloudy swelling and hydropic degeneration were detected in the liver section of all the hamsters inoculated with the isolates and the positive control (Plate IV) except those inoculated with L. biflexa and the human isolate, vv7JH. Leptospires were demonstrable in the kidney sections of all the inoculated hamsters except those inoculated with L. biflexa (Plate V) by the fluorescent antibody technique (Plates VI-X). The number of fluorescing leptospires varied in the different kidney sections.

The results of the biological characteristics of the isolates are presented in Table 6.6. Six of the isolates reacted identically with the positive control (pomona) but the reaction of the human isolate vv7JH was variable. This human isolate was inhibited by 8-azaguanine at a concentration of

225µg/ml and was resistant to normal fresh serum. Only L. biflexa was susceptible to the lytic action of normal fresh serum.

The restriction patterns of the isolates and other members of the Pyrogenes and Sejroe serogroups were comparable (Plates XI - XIII). The restriction patterns of the five bovine isolates represented by vv2JA, vv3JA and vv4JA in the REA were identical but different from other serovars within the Pyrogenes serogroup (Plate XI). The human isolate, vv7JH showed an extra band pattern but the restriction patterns of vv1JA and the reference hardjobovis genotype were identical (Plates XII and XIII).

Table 6.1: Microscopic agglutination test (MAT) result of reference strains and Nigerian isolates using vv3JA hyperimmune serum

Antigen	Reciprocal titre
pyrogenes Salinem	1600
hamptoni Hampton	25600
robinsoni Robinson	6400
alexi HS 616	6400
quaratuba An 7705	0
kwale Julu	25600
princestown TVRL 112499	6400
varela LT 992	0
zanoni Zanoni	12800
abramis Abraham	1600
biggis Biggs	3200
manilae LT 398	1600
myocastoris LSU 1551	1600
cenopa MW2	100
bagua MW12	12800
camlo LT64-67	3200
vv1JA (Nigerian isolate)	0
vv2Ja " "	25600
vv3JA " "	25600
vv4JA " "	25600
vv5JA " "	25600
vv7JH " "	0
vv8JA " "	25600

Table 6.2: Microscopic agglutination test (MAT) result of reference strains and Nigerian isolates after absorption of vv3JA hyperimmune serum with *kwale* Julu

Antigen	Reciprocal Titre
kwale Julu	0
pyrogenes Salinem	0
hamptoni Hampton	0
robinsoni Robinson	0
princestown TVRL	0
zanoni Zanoni	0
biggis Biggs	0
manilae LT 398	0
bagua MW12	0
camlo LT 64-67	0
vv1JA (Nigerian isolate)	0
vv2JA " "	6400
vv3JA " "	6400
vv4JA " "	6400
vv5JA " "	6400
vv7JH " "	0
vv8JA " "	6400

Table 6.3: Microscopic agglutination test (MAT) result of Sejroe reference strains and Nigerian isolates using vv1JA hyperimmune serum

Antigen	Reciprocal Titre
balcanica 1627 Burgas	3200
caribe TVRL 61866	0
geyaweera Dikkeni	100
gorgas 1413U	200
haemolytica Marsh	0
hardjo Hardjoprajitno	12800
hardjo Hardjobovis	12800
istrica Bratislava	100
medanensis Hond HC	1600
nyanza Kibos	6400
polonica 493 Poland	0
recreo 380	0
ricardi Richardson	100
roumanica LM 294	0
saxkoebing Mus 24	0
sejroe M84	200
trinidad TRVL 34056	12800
wolfii 3705	3200
vv1JA (Nigerian isolate)	12800
vv2JA " "	0
vv3JA " "	0
vv4JA " "	0
vv5JA " "	0
vv7JH " "	0
vv8JA " "	12800

Table 6.4 Microscopic agglutination test (MAT) result of reference Sejroe serogroup strains and Nigerian isolates after absorption of vv1JA hyperimmune serum with hardjo Hardjoprajitno

Antigen	Reciprocal titre
balcanica 1627 Burgas	800
hardjo Hardjoprajitno	3200
hardjo Hardjobovis	3200
nyanza Kibos	100
sejroe M84	400
wolfii 3705	400
medanensis Hond HC	100
vv1JA Nigerian isolate	3200
vv2JA " "	0
vv3JA " "	0
vv4JA " "	0
vv5JA " "	0
vv7JH " "	3200
vv8JA " "	0

Table 6.5: Serological response of hamsters inoculated with bovine and human leptospiral isolates

Isolate number[a]	Mean microscopic agglutination test(MAT) titres post-inoculation			
	Day 0	Day 7	Day 14	Day 21
vv1JA	0	0	1:20	1:20
vv2JA	0	0	1:80	1:100
vv3JA	0	0	1:80	1:80
vv4JA	0	0	1:80	1:80
vv5JA	0	0	1:100	1:100
vv7JH	0	0	0	0
vv8JA	0	0	1:80	1:80
L. pomona (Reference strain)	0	0	1:100	1:100
L. biflexa (Reference strain)	0	0	0	0

[a]All isolates are of bovine origin while vv7JH is of human origin.

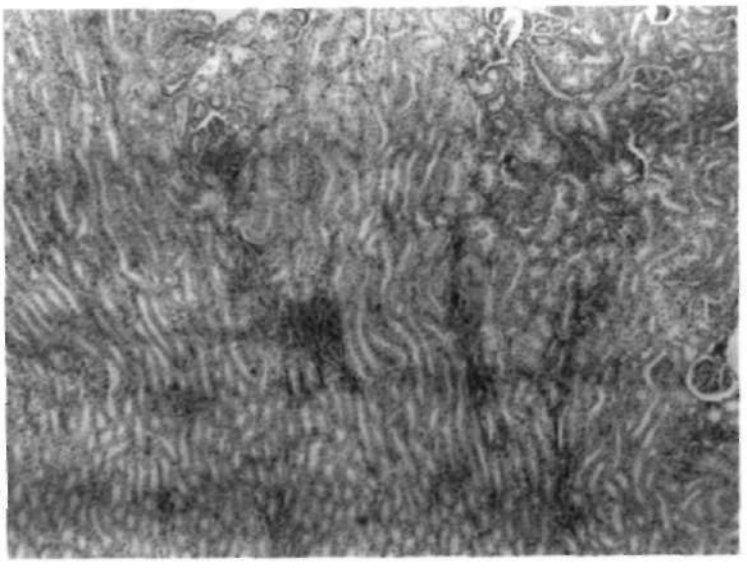


PLATE 1: MODERATE CONGESTION AND AGGREGATION OF MONONUCLEAR CELLS AROUND TUBULES AND BLOOD VESSELS. x63.

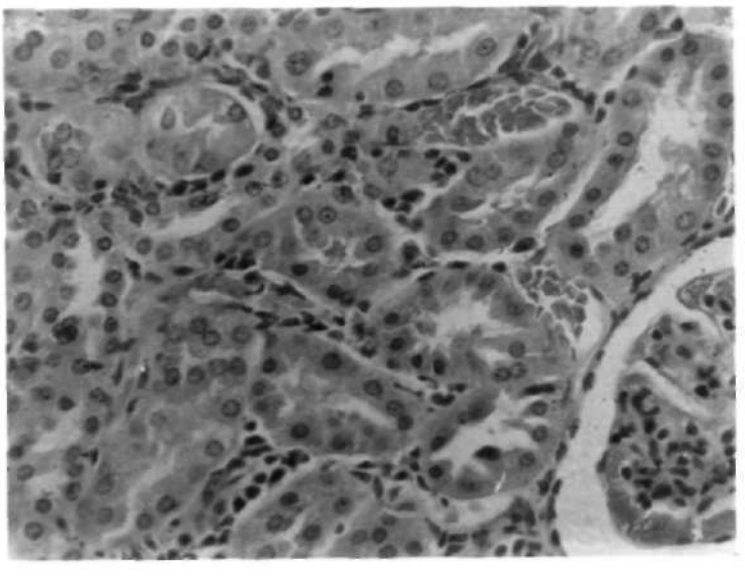


PLATE II: CONGESTION AND AGGREGATION OF MONONUCLEAR CELLS AROUND TUBULES AND BLOOD VESSELS. x400.

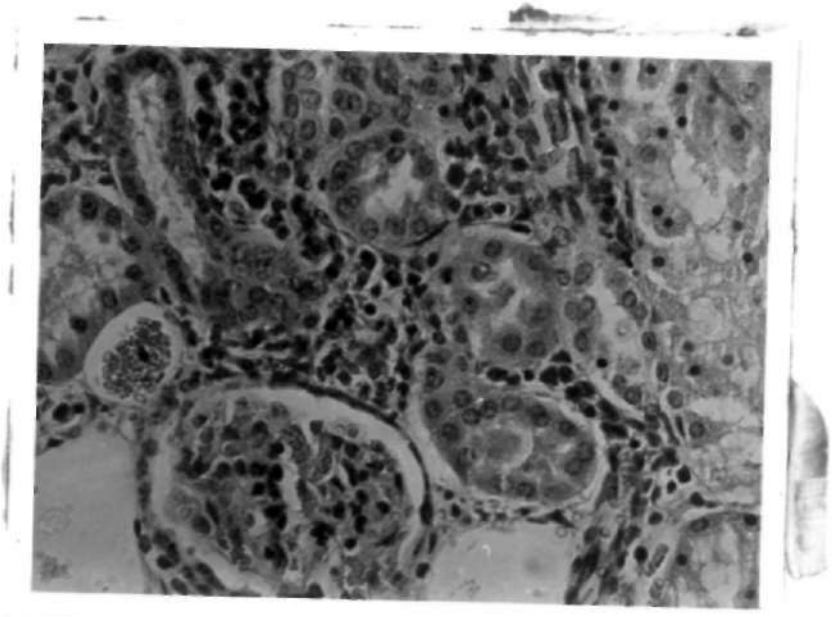


PLATE III: CONGESTION IN THE CORTEX AND MEDULLA, AGGREGATES OF MONONUCLEAR CELLS. x400.

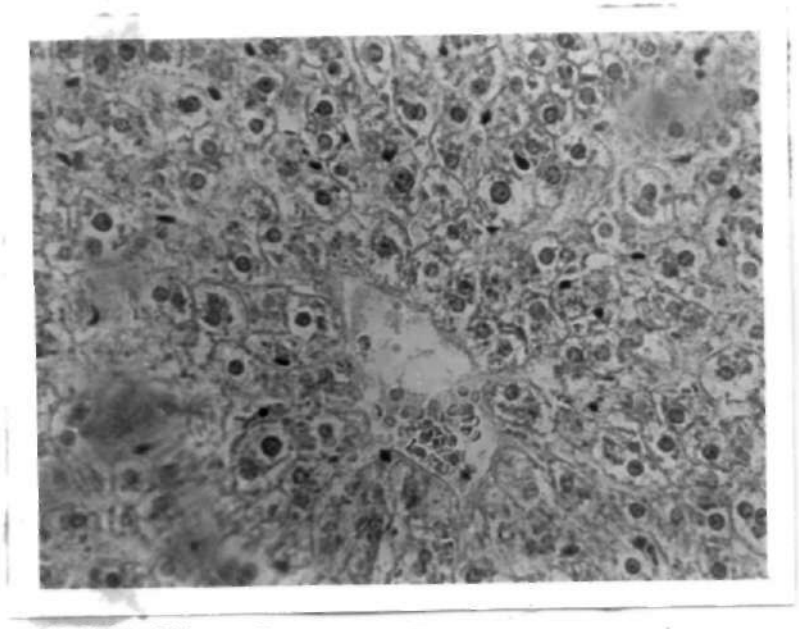


PLATE IV: CLOUDY SWELLING AND HYDROPIC DEGENERATION IN THE LIVER. x400.



PLATE V: FA - STAINED KIDNEY SECTION OF HAMSTER INOCULATED WITH LEPTOSPIRA BIFLEXA (NEGATIVE CONTROL).

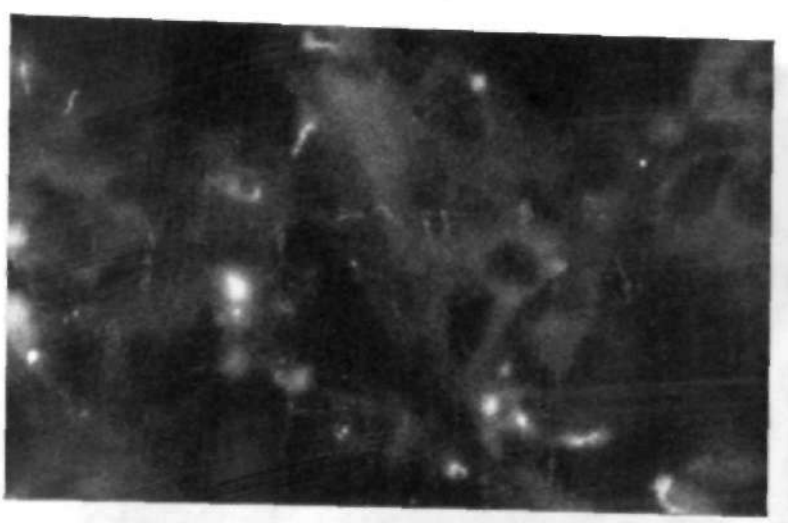


PLATE VI: FA - STAINED KIDNEY SECTION OF HAMSTER INOCULATED WITH LEPTOSPIRA POMONA (POSITIVE CONTROL).

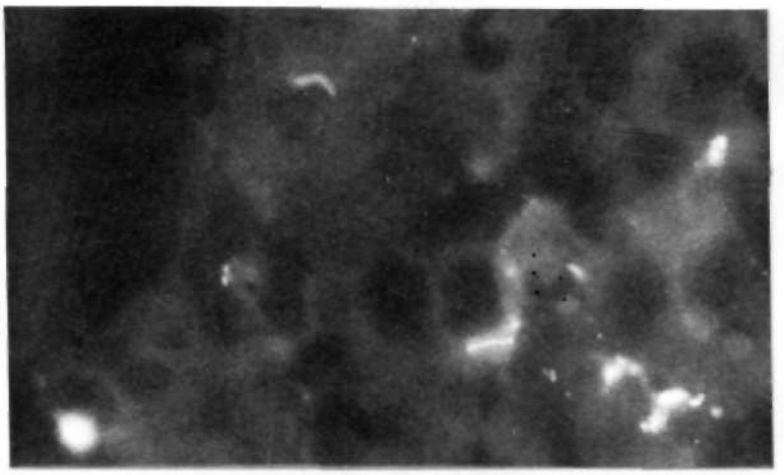


PLATE VII: FA - STAINED KIDNEY SECTION OF HAMSTER INOCULATED WITH BOVINE ISOLATE VV1JA.

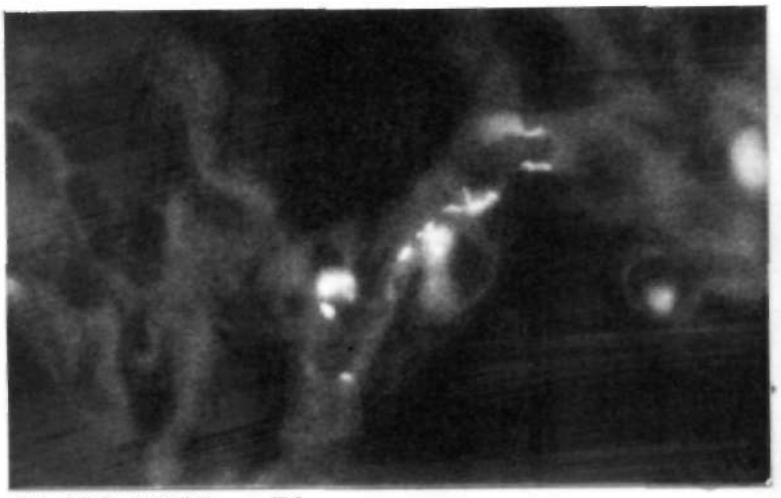


PLATE VIII: FA - STAINED KIDNEY SECTION OF HAMSTER INOCULATED WITH BOVINE ISOLATE VV2JA.

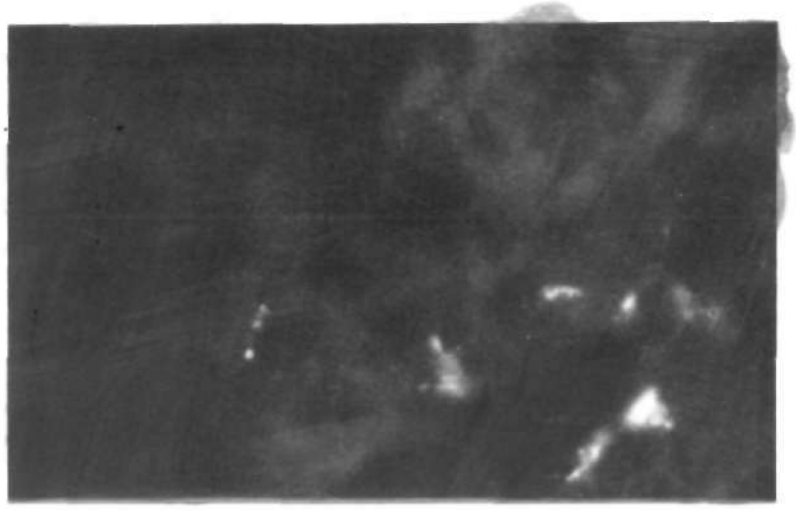


PLATE IX: FA - STAINED KIDNEY SECTION OF HAMSTER INOCULATED WITH BOVINE ISOLATE VV5JA.

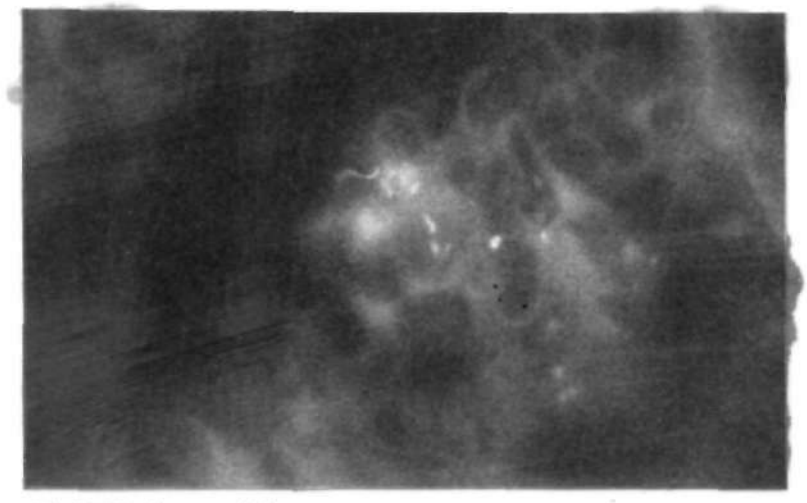


PLATE X: FA - STAINED KIDNEY SECTION OF HAMSTER INOCULATED WITH HUMAN ISOLATE VV7JH.

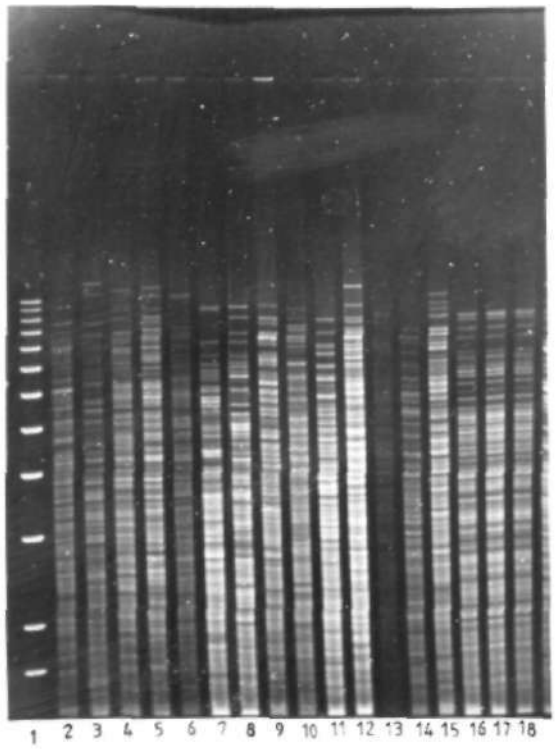
Table 6.6 Biological characteristics of leptospires isolated from cattle and man in Plateau State of Nigeria

Isolate number	Re-isolation from hamster kidney	Viability and growth at 13°C	Growth inhibition 8-azaguanine at 225µg/ml	Conversion of cells to spherical forms in 1M NaCl	Resistance to normal fresh serum
vv1JA	+	-	+	+	+
vv2JA	+	-	+	+	+
vv3JA	+	-	+	+	+
vv4JA	+	-	+	+	+
vv5JA	+	-	+	+	+
vv7JH	-	+	+	-	+
vv8JA	+	-	+	+	+
L. pomona (Reference strain)	+	-	+	+	+
L. bi[illegible] (Reference strain)	-	+	-	-	-

+Strain positive for characteristic

-Strain negative for characteristic

- EcoRI digest, - 20cm 0·6% agarose gel run for 20h at 65 Volts
Lane 1 - molecular weight markers
2 - abramis - Abraham
3 - alexi - Hs 616
4 - biggis - Biggs
5 - camlo - LT 64-67
6 - guaratuba - An 7705
7 - hamptoni - Hampton
8 - kwale - Julu
9 - manilae - LT 398
10 - myocastoris - LSU 1551
11 - princestown - TRVL 112499
12 - pyrogenes - Salinem
13 - robinsoni - Robinson
14 - varela - 1019
15 - zanoni - Zanoni
16 - VV 2 JA
17 - VV 3 JA
18 - VV 4 JA



PYROGENES SEROGROUP

PLATE XI: RESTRICTION ENDONUCLEASE ANALYSIS (REA) PATTERNS FROM REFERENCE STRAINS IN PYROGENES SEROGROUP AND BOVINE ISOLATES DIGESTED WITH EcoRI.

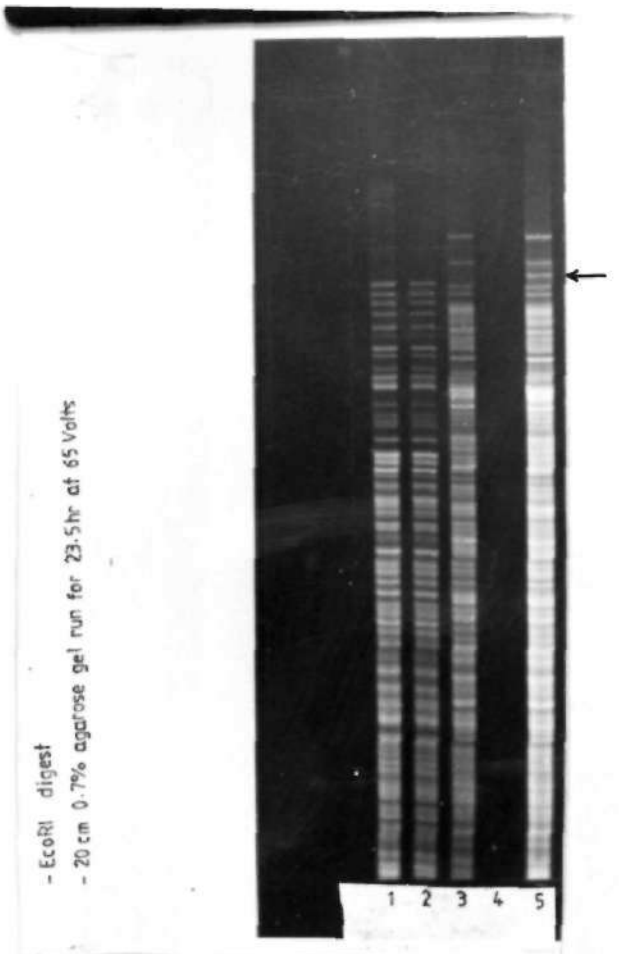


PLATE XII: RESTRICTION ENDONUCLEASE ANALYSIS (REA) PATTERNS FROM HARDJO REFERENCE GENOTYPES AND ISOLATES DIGESTED WITH EcoRI.

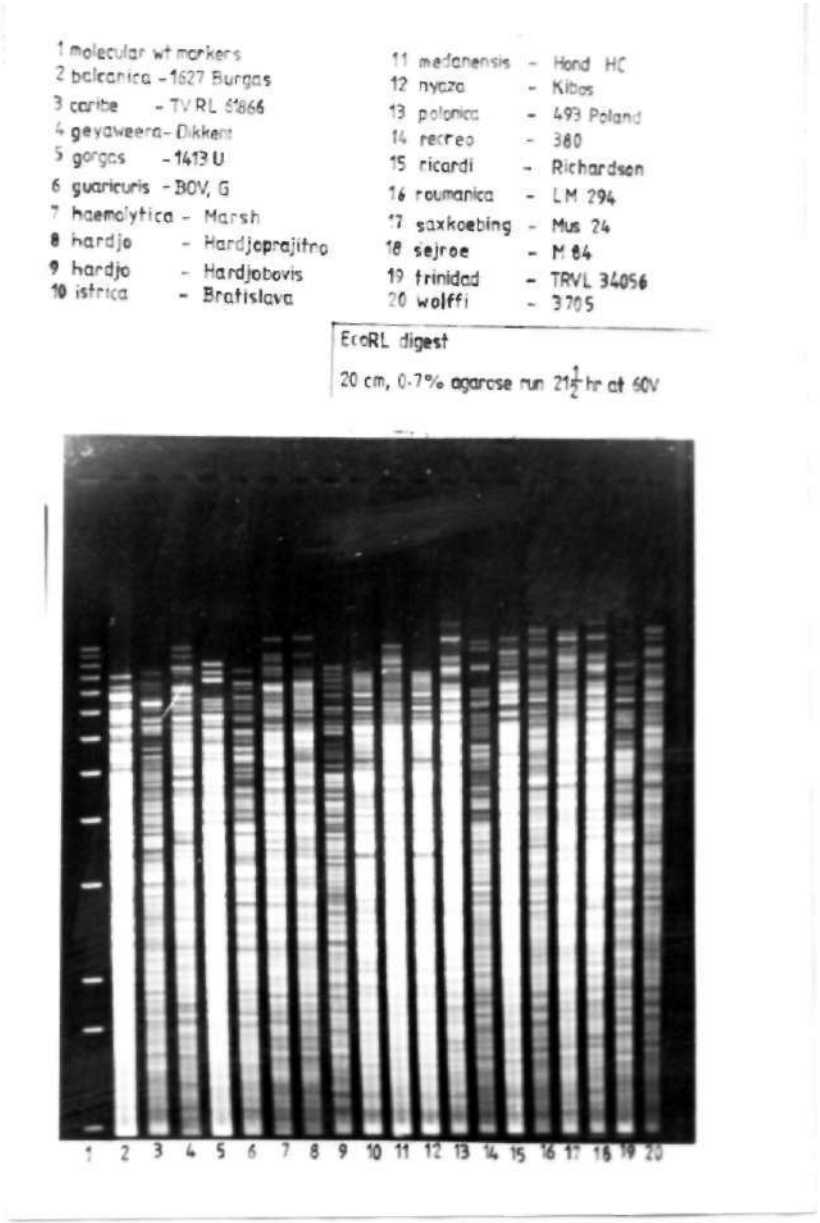


PLATE XIII: RESTRICTION ENDONUCLEASE ANALYSIS (REA) PATTERNS OF CHROMOSOMAL DNA FROM REFERENCE STRAINS IN THE SEJROE SEROGROUP DIGESTED WITH EcoRI.

6.4 Discussion

Specialized serological tests like cross-agglutinin absorption test and factor analysis are time, labour and material intensive and are therefore best performed in reference laboratories (Turner, 1968; Dikken and Kmety, 1978). The serological reactions of the isolates with the reference serovars indicated that the isolates belonged to two distinct serogroups. The high titre obtained with vv7JH, vv1JA and reference serovars hardjo and seiroe probably reflects the close antigenic relatedness of these serovars. Although the five bovine isolates reacted equally at high titre of 1:25,600 with serovars kwale and hamptoni, no titre to these reference serovars were detected after cross-absorption with kwale. The implication of this result was that all the five isolates possessed a separate antigen which was absent in other members of the Pyrogenes serogroup, thus indicating possibly the isolation of a new serovar in Nigeria.

The absence of clinical manifestation in all the inoculated hamsters including the positive control was unexpected because weanling hamsters are the best animal model for experimental leptospirosis (Sulzer and Jones, 1974). In a similar experiment using guinea pigs, mice, voles and hamsters, Orr and Little (1979) stated that none of the laboratory animals showed any febrile or clinical response to inoculation with leptospiral strains although all species used excreted leptospires in their urine for variable periods between 20 and 30 days after inoculation. Apart from the human isolate, all other isolates were re-isolated in pure cultures from the kidney samples of the inoculated hamsters. This failure to re-isolate may be due to low pathogenicity of serovar hardjo to laboratory animals (Sullivan, 1970). Although hamsters have been shown to be effective for the recovery of new leptospiral isolates (Myers and Jelambi, 1975), direct culturing of isolates in bacteriological media was reported a better method than isolation from hamster kidney (Herr et al. 1982). The antibody titres elicited by the isolates (1:20 - 1:100) were lower than 1:1000 (Sullivan, 1970) and 1:1600 (Orr and Little, 1979) earlier reported. The low titre of 1:20 observed in vv1JA-inoculated hamster was not surprising because

serovar hardjo usually does not evoke high antibody titres (Ellis, 1984). Herr et al. (1982) reported negative serology in hamsters infected with L. pomona and attributed the failure to early termination of their experiment. In the present study, vv7JH did not elicit any serological response at a titre of 1:20 up till 21 days post-inoculation when the study was terminated.

The histopathological changes observed in the kidney and liver sections of the inoculated hamsters were not specific and consistent enough to be of any diagnostic significance. Marked hyperaemia, haemorrhages, tubular degeneration, cast formation and inflammatory cell infiltration described by Fuzi and Csoka (1962) were not demonstrable probably due to decrease in virulence of the isolates and the pomona reference serovar used. All the isolates used in the present study had been subcultured several times to keep them alive and check their purity before characterization. Faine (1957) showed that severity of lesions correlated with the number of leptospires present in the tissue. Leptospires were demonstrated by immunofluorescence in the kidney tissue sections of all the hamsters including those inoculated with the human isolate, vv7JH. In an

experimental serovar hardjo infection in calves, Ellis et al. (1986) observed that FA-positive, culture-negative cases were usually the result of the overgrowth of cultures by contaminating microorganisms. That observation was not valid in the present study because all the cultured media remained uncontaminated till the termination of the experiment. It is possible that the vv7JH seen in the FA-stained kidney section were dead leptospires which managed to multiply in the kidney tissue before being killed by the body defence mechanisms in the inoculated hamster. This explanation may also account in part for the failure of vv7JH to elicit any serological response since infection was hardly established.

The biological characteristics of all the bovine isolates were consistent with those of pathogenic species, Leptospira interrogans (Johnson and Faine, 1984). The human isolate, vv7JH possessed biological characteristics of both pathogenic and saprophytic leptospires. Cinco et al. (1988) described a leptospiral strain, K6196, which was inhibited by 8-azaguanine and failed to grow at 13°C but was not converted to spherical form in 1M NaCl. In another experimental study with two strains of L.

andamana which were isolated from man, Johnson and Muschel (1966) observed that both strains possessed some physiological characteristics intermediate between the saprophytes and the pathogens.

The REA patterns clearly showed that five of the bovine isolates were identical but were definitely different from other serovars within the Pyrogenes serogroup. The extra band observed in the restriction pattern of vv7JH when compared with the restriction pattern of hardjoprajitno genotype, could be a result of genetic variation although Thiermann et al. (1985) had stated that the genome of leptospires was genetically stable. However, similar antigenic variants had been reported in leptospires cultured from the blood of experimentally-infected puppies and pigs (Fukui et al., 1980), mice (Yanagawa and Adachi, 1978) and guinea pigs (Yanagawa et al., 1979).

The isolation and identification of bovine and human genotypes of serovar hardjo is significant. These two genotypes are increasingly being recognized as the commonest cause of bovine and human leptospirosis in many parts of the world. The isolation of a possible new serovar in the Pyrogenes serogroup clearly shows that the true prevalence of leptospirosis in Nigeria can only be ascertained when more leptospires are isolated and identified.

SUMMARY AND CONCLUSION

Serum samples obtained from 1,537 cattle, 710 human volunteers and 105 trapped rodents in Plateau State of Nigeria were screened for the presence of leptospiral agglutinins using 13 serovars in a modified microscopic agglutination test. The results of the survey indicated that 14.4 per cent of cattle, 18 per cent of human and 44.8 per cent of rodents were serologically positive for leptospirosis at a titre of 1:100 and above. The significance of high serological prevalence in all the species examined with low isolation rate is yet to be determined since the epidemiology of leptospirosis in Nigeria is poorly understood.

In a bacteriological examination of bovine and rodent kidneys in Plateau State, six strains of leptospires were isolated. Five of the isolates were serologically and genetically identical and they belonged to a completely new serovar in the Pyrogenes serogroup. The sixth strain was identified as serovar hardjo, genotype hardjobovis. No leptospire was isolated from any of the rodent kidneys. A leptospiral variant of strain Hardjoprajitno was also isolated from the mid-stream urine of an abattoir worker in Jos, Plateau State.

It was established in this study through serological and bacteriological procedures that leptospirosis exists in animals and man in Nigeria. Owing to the high reactor rate and the isolation of new serovars in the present study as well as previous isolation of leptospires from cattle and rodents by other workers, it is concluded that leptospirosis is endemic in Nigeria. The isolation of serovar hardjo poses serious threat to cattle industry in Nigeria and this development calls for both State and Federal Government agencies to take immediate and adequate measures to control this cattle-adapted serovar in the country. The serovar is reported to be responsible for most bovine and human leptospirosis in many parts of the world. Clinical infection due to serovar hardjo is often associated with sudden onset agalactia in adult cattle, abortion, stillbirth, birth of weak calves and infertility.

The following points are therefore suggested to improve future studies of leptospirosis in Nigeria:

1. Deliberate efforts be made to obtain and identify more serovars prevalent in livestock, man and his pets, wildlife, rodents and contaminated environments. Knowledge of this

will provide a basis for the development of potent vaccines for use in animals to control the disease;

2. The use of 1:100 as the screening cut-off titre in the microscopic agglutination test is no longer realistic since it has been demonstrated that serovar hardjo which does not usually elicit high antibody titre in infected host is present in Nigeria. Future epidemiological surveys should consider titres lower than 1:100 as significant;

3. It is important to educate people at risk especially those engaged in abattoir and farm operations on the dangers of contracting leptospirosis;

4. Physicians, veterinarians and laboratory diagnosticians should routinely consider leptospirosis in the differential diagnoses of all icteric, febrile illnesses, agalactia, abortion, infertility and decreased productivity in Nigeria. Patience and care are also required by laboratory diagnosticians when handling leptospirosis suspected specimens;

5. Similar studies should be undertaken in other States of the federation including the Federal Capital Territory to compare results;

6. There is need to further characterize all the isolates obtained in this present study to determine their appropriate serovars or give them new names if necessary. To do this will depend on the cooperation of the Reference laboratories and the International Committee on Systematic Bacteriology, as this is beyond the capability of any individual and any laboratory in Nigeria for now;

7. There is need by the Federal Government to establish and equip a research laboratory on the studies of leptospirosis and its agents in Vom, Nigeria, where the personnel and techniques are already existing.

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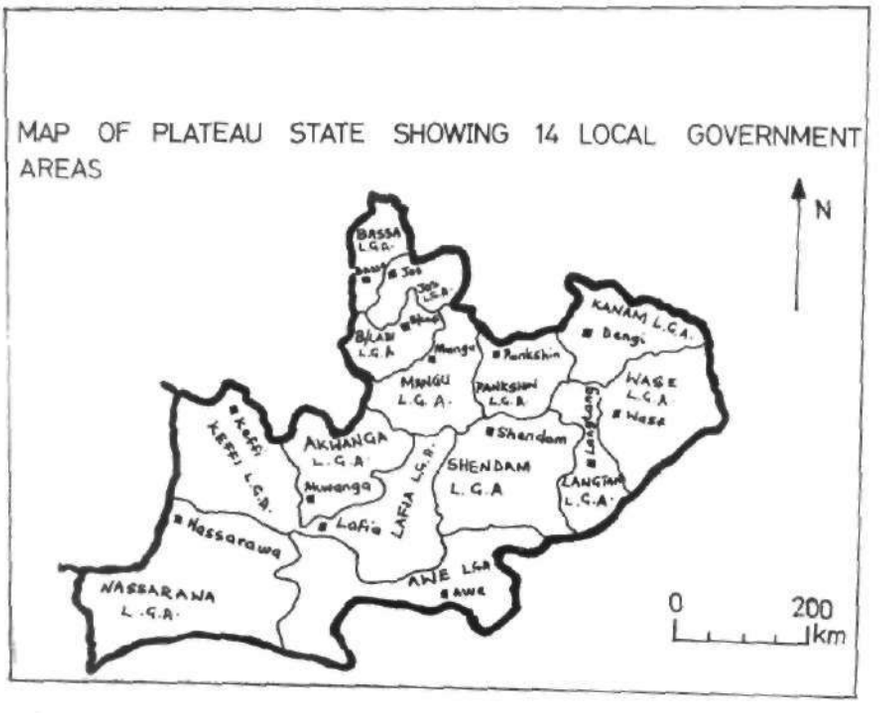
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APPENDIX I



Map of the 14 Local Governement Areas of Plateau State.

APPENDIX 2

Questionnaire on Bovine Leptospirosis

Owner's name and address _____

_____

Local Government Area (L.G.A.) _____

Date _____

Number of cattle in the herd _____ No. sampled __

Breed of cattle _____

Type of establishment /Govt./ /Settled/ /Nomadic/

If settled, for how long? _____

Cases of abortion /___/, mastitis /___/, stillbirth /___/, infertility observed in the herd during the past one year.

Any sick animals in the herd at present? _____

What disease/s suspected? _____

What is the usual treatment given? /By self/ /By veterinary personnel/

Other animals kept in the same farm _____

Source of water supply /piped/ /stream/ /well/ /pond/

No. of rodents seen or killed in the farm premises.

Any icterus observed in cattle or man in the farm?

Animals and pets sharing habitation with man in the farm. What are the commonest diseases observed in the farm? _____

What preventive measures are taken against disease /vaccination/ /none/

APPENDIX 3

Questionnaire on Human Leptospirosis

Name and address of blood donor _____

_____

Hospital/Health Centre, L.G.A. _____

Age and Sex _____

Occupation _____

For how long on the job? _____

Clinical history _____

_____

Degree of contact with animals _____

List the animals _____

Any on-going pyrexia _____

Any headache, muscle pain? (Describe) _____

_____

What treatment usually given? _____

Any recreation involving surface water, pond or water-logged soil? _____

Any rodents seen or handled in the house? _____