"A STUDY ON LEPTOSPIROSIS"

by

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A dissertation submitted in partial fulfilment of the requirements for the degree of Masters of Science (Medical Microbiology) in the University of London.

LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE.

1984

CONTENTS

GENERAL INTRODUCTION

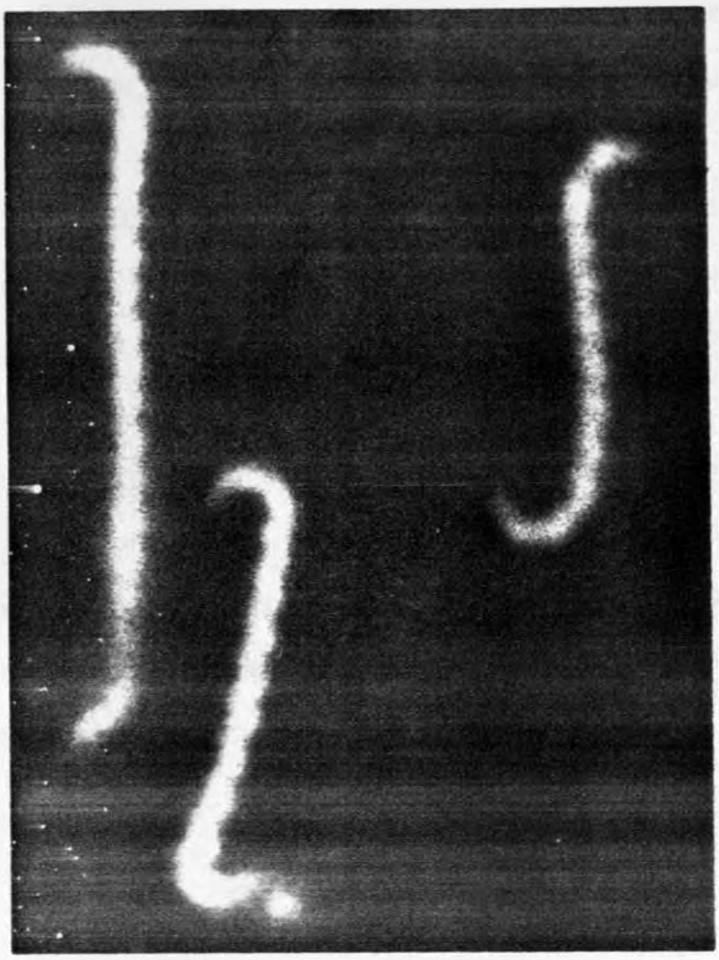
(1) TAXONOMY

Leptospires were first observed and described by Stimson in 1907. Leptospires are fine spiral-shaped bacteria with characteristic morphology seen by dark ground, phase contrast, fluorescent and electron microscopy. The organisms appear straight with one or both ends hooked. Their diameter is about 0.1μm and their length varies from 6μm to 20μm *(see Fig. 1)*.

Electron-microscopical studies of leptospires have revealed the following features; (1) the peripheral structure is a multilayered outer envelope or membrane; (2) enclosed by this outer envelope is a helical protoplasmic cylinder which consists of the peptidoglycan layer, cytoplasmic membranes and the cytoplasmic contents of the cell; (3) the two peripheral flagella (axial fibrils) lie between the outer envelope and the protoplasmic cylinder and are attached to the latter at sub-terminal positions. The free ends of the periplasmic flagella extend towards the cell centre but rarely overlap. The helical conformation of leptospira is right-handed (clockwise coiling). *(Johnson, R.C., 1977, 1981, Johnson, R.C. and Faine, S. 1983)*.

The genus Leptospira was created by Noguchi in 1917 and belongs to the family Leptospiraceae of the order Spirochaetales *(Johnson, R.C., 1984)*. Although DNA-DNA annealing tests indicate at least seven distinct genetic groups exist within the genus, only two species; *L.biflexa* (representing saprophytic and free living strains) and *L.interrogans* (representing parasitic and potentially pathogenic strains) are recognised. Within each strain are serovars, the basic taxon, which are arranged according to their antigen interrelationships demonstrated by micro-agglutination and absorption reactions with rabbit sera *(W.H.O., 1982)* Currently, there are 20 serogroup in the *L.interrogans* strains with 180 serovars. *L.biflexa* contains 38

FIGURE 1. Illustrates the characteristic morphology of leptospire. Note the hooked ends and spiral shape of the organism. Magnification X 3000.



serogroups with corresponding 65 serovars *(see Table I and W.H.O., 1982)*. The genus specific antigen is found in serogroup *Semaranga serovar Patoc*. The term serogroup has no taxonomic significance.

Although the free-living *L.biflexa* and the parasitic *L.interrogans* are indistinguishable morphologically, several tests are available for differentiating between them. Serovars of *L.interrogans* are rapidly converted to spherical forms in hypertonic environments, whereas *L.biflexa* is relatively resistant to this morphological alteration. *L.interrogans* does not grow at 13°C or in the presence of 225 µg/ml 8-azoguanine, while *L.biflexa* does. *(Johnson, R.C., 1977, 1981, Johnson, R.C., and Faine, S., 1983)*.

Classification of the *L.interrogans* serogroup is based on cross-absorption methods. It is tedious, time consuming and technically difficult. *(Turner, L.H., 1967, 1968a, b)*. Newer methods, such as bacterial restriction endonuclease DNA analysis (BRENDA) have been used with accuracy and ease to differentiate the various serogroups of *L.interrogans*. *(Robinson et al., 1982)*. The use of such modern analysis systems will lead to more accurate and reproducible classification, and a more precise epidemiological understanding of the relationship between strains and their hosts.

TABLE 1. Recognized serogroups of leptospira.

(W.H.O., 1982)

L.interrogans var:	*L.biflexa var:*	
Icterohaemorrhagiae	*Abaete*	
Javanica	*Ancona*	*Ondina*
Celledoni	*Andamana*	*Orvenco*
Pyrogenes	*Aurisina*	*Parapatan*
Ballum	*Basrich*	*Percedol*
Autumnalais	*Basovizza*	*Poona*
Grippotyphosa	*Bessemans*	*Pulpudeva*
Hebdomadis	*Cadore*	*Ranarum*
Sejroe	*Camtchia*	*Semaranga*
Saxkoebing	*Cau*	*Sidonia*
Wolffi	*Codice*	*Sobradinoho*
Mini	*Dindio*	*Tevere*
Bataviae	*Doberdo*	*Tharcia*
Tarassovi	*Garcia*	*Tororo*
Pomona	*Holland*	*Udine*
Cynopteri	*Khoshamian*	*Vinzent*
Canicola	*Lazio*	
Australis	*Malomievo*	
Panama	*Maritza*	
Djasiman	*Muggia*	
Shermani	*Nomentano*	

(II) ECONOMIC ASPECT

It is probable that leptospirosis is the most widespread zoonosis in the world *(van der Hoeden, 1964)*. It is geographically widespread producing a wide range of clinical manifestations of varying severity which may be undiagnosed or overdiagnosed. In countries such as Australia where studies have been done, it is estimated that 1 in every 10 dairy farmers and meat workers and 1 in every 4 meat inspectors, is likely to acquire leptospirosis in a working life time of 30 years. *(Faine, S., 1983)*. In 1980, Little and his colleagues estimated that about 10% of bovine abortions in S.W. England were due to leptospirosis, whereas in 1954 the United States government estimated more than 100 million dollars lost annually attributable to leptospirosis. In 1959, the World Health Organization noted that bovine leptospirosis was a major disease rivalling brucellosis in certain countries. *(W.H.O., 1959)*.

Zoonotic diseases have direct consequences for national economic development. They reduce the available supply of much needed high protein food. The economic costs incurred in the control of zoonotic diseases in animals are often enormous and are in addition to medical costs and loss of work. Additional expenses arise from surveillance mechanisms for detecting animal reservoirs.

(III) EPIDEMIOLOGY

The natural reservoir for Leptospira is wild animals, particularly the rodent family. Domestic animals such as farm livestock and dogs may also become infected, either indirectly by contact with infected urine from the above host reservoir, or directly with leptospiral serogroups specific for their species, e.g. serogroup *Hebdomadis serovar hardjo* (now re-classified as serogroup *Wolffi serovar hardjo*. *(ICSB-ST of Leptospires, 1982)* affects cows and *var Canicola*, dogs.

Rodents are the main carriers of *L.interrogans var Icterohaemorrhagiae*. This organism causes classical Weil's Disease and in Great Britain, 32% of all human cases of leptospirosis in 1983 were due to this leptospiral serogroup, *(Table II)*. The host animal is often asymptomatic but carries the leptospires in its kidneys and excretes the organism in high numbers in the urine, contaminating the surrounding environment, usually riverside waters and animal feedstuffs on farms. Human infection results from direct or indirect exposure to this infected urine. Leptospires enter the body through cuts and abrasions of the skin or via the mucous membrane linings of the nose, mouth or eyes.

TABLE II. Human Leptospirosis in the British Isles, 1978 to 1983.

(CDR., 79/21,81/11,82/11,83/31, Waitkins and Wanyangu, 1984).

	1978	1979	1980	1981	1982	1983
Icterohaemorrhagiae	26 (40%)	23 (41%)	27 (56%)	39 (54%)	23 (37%)	37 (32%)
Hebdomadis (hardjo)	33 (51%)	18 (33%)	13 (27%)	18 (25%)	13 (21%)	51 (45%)
Canicola	5 (8%)	5 (9%)	2 (4%)	4 (6%)	7 (11%)	6 (5%)
Others	1 (1%)	9 (17%)	6 (13%)	11 (15%)	19 (31%)	20 (18%)
TOTAL NUMBER	65	55	48	72	62	114

The predominating infecting serogroups found in this country are: *L.interrogans var Icterohaemorrhagiae; var Canicola and var Hebdomadis serovar hardjo* (now re-classified as *Wolffi serovar hardjo. ICSB-ST of Leptospires, 1982). (Table III).*

TABLE III. Human Leptospirosis, British Isles, 1978 to 1983.

(CDR, 79/21,81/11,82/11,83/31, Waitkins and Wanyangu, 1984.;

Occupation	*Icterohaemorrhagiae*	*Hebdomadis (hardjo)*	*Canicola*
Farmer	50	113	1
Abattoir - Meat Worker	2	6	0
Vets	1	3	0
Miner	2	0	0
Sewer Worker	4	0	0
Other risks			
Water	40	4	0
Rats	25	0	2
Dogs	0	0	9
Misc., not stated	48	18	17
TOTAL NUMBER:	172	144	29

In the past occupations associated with water or sewage were at particular risk from Weil's Disease. This group worked in rat infested conditions, in water often polluted with Leptospira - contaminated urine. Any cuts or abrasions were easily infected with leptospira, therefore, not surprisingly, leptospirosis was quite common in sewer workers. However, with modern post control measures and the use of protective clothing, the risk to sewer men has diminished dramatically. Table III shows that during the past six years, between 1978 and 1983, only four cases of leptospirosis were found in this group of workers. The major occupational risk today is among workers in the farming industry. 50 of a total of 172 cases of

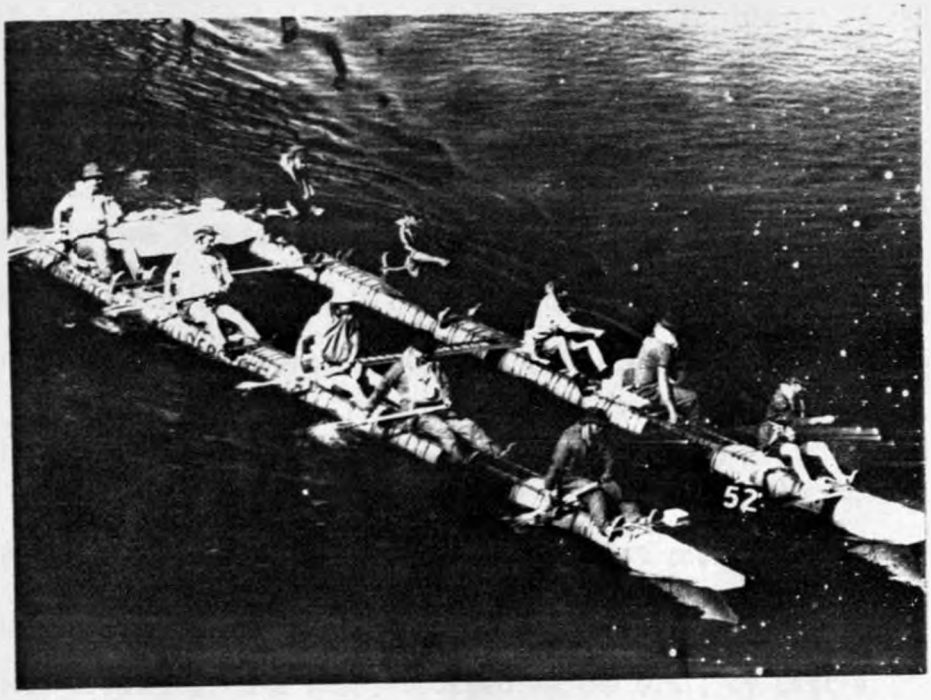
Icterohaemorrhagiae infections occurred in the farming group. Similar protective measures as those introduced for sewermen would be difficult to implement because of the nature of farming practices. Nevertheless, awareness of the risks involved is often a good prophylactic measure.

During the last decade there have been several reports from the U.S.A. showing the increasing frequency of leptospirosis due to water associated leisure activities. This trend had also been noted in this country. Table III shows that 40 cases of leptospirosis due to *Icterohaemorrhagiae* were linked with water exposure. Most of these were bathers in fresh water ponds and streams, some from canoeists performing eskimo rolls, for instance during 1983, 7 such cases were reported. A recent increase of leptospiral infections in people pursuing the latest leisure craze - "Raft Racing" has also been noted, this is a most competitive sport where cuts and bruises are easily sustained with the consequent danger of infection. Indeed, leisure activities account for the major proportion of Weil's Disease in Great Britain *(Waitkins, S., 1983 and Fig.2)*.

Leptospiral infections due to the serogroup *Canicola* are usually isolated incidents and do not contribute greatly to the total number of infections in this country. Dogs, of course, can become infected, often without any clinical signs but compared to the dog population of Great Britain, the numbers recorded makes *Canicola* infections insignificant. The low incidence of leptospirosis in dogs is probably due to the rigorous vaccination programmes which exist in Great Britain.

The new emerging hazard is undoubtedly those infections associated with cattle, Cattle Associated Leptospirosis (CAL). This particular infection usually presents as a flu-like illness (see below) and is due to the serogroup *Hebdomadis serovar hardjo* (now re-classified as

FIGURE 2. This shows the competitive nature of raft racing. Cuts and abrasions can easily be sustained and leptospires may penetrate through these into the body.



(Reproduced by kind permission of The Hereford Times PLC)

serogroup *Wolffi serovar hardjo, ICSB-ST of leptospires, 1982).* During the years 1968 to 1972, one third of all bovine serum submitted to the Central Veterinary Laboratory, Weybridge, had serological evidence of leptospirosis using the microscopic agglutination test as a screening method. Over 90% of these were due to the *Hebdomadis* serogroup *(Report 1972).* It soon became apparent to veterinarians that approximately 10% of all cattle abortions were associated with high levels of leptospiral antibodies. They found that 96% of the recorded positive cases could be attributed to a single leptospire - *Hebdomadis serovar hardjo (Little et.al.,1980a).* Supporting work was also published by Ellis and his colleagues in Northern Ireland *(Ellis et.al.,1982),* these workers also reported that 42% of randomly selected aborted bovine fetuses and 69% aborted fetuses from farms with known abortion problems could be attributed to *Hebdomadis serovar hardjo* (now re-classified as serogroup *Wolffi serovar hardjo).* The same pattern of disease has been described in Australia, New Zealand and Israel. Indeed, the New Zealand survey by Hellstrom and Blackmore estimated that nearly 90% of all cattle herds in that country showed serological evidence of infection *(Hellstrom and Blackmore, 1980).* Lower figures of about 30% have been reported in the U.K., by Little and his colleagues, and Ellis and his associates. *(Little, et.al.,1980 and Ellis, et.al.,1982b).*

It is therefore, not surprising that such evidence of epidemic proportions of leptospirosis in cattle should also be reflected in the incidence of Cattle Associated Leptospirosis in Man.

Coghlan and her colleagues reported that in the three years 1978 to 1980 there were 64 cases of Cattle Associated Leptospirosis; they maintained that the *Hebdomadis* serogroup accounted for 37% of all cases of leptospirosis in the U.K. and over 50% of such cases were positively proven to be due to the serovar *hardjo*. In 1983, 45% of leptospiral

infections in the U.K. were due to the serovar *hardjo*, the majority of patients were cowmen or farmers who regularly milked their cows (80%), see Table II and IV.

TABLE IV. Incidence of serogroup *Hebdomadis serovar hardjo* in 1983 (Leptospira Reference Laboratory, Hereford)

OCCUPATION (Farming)	Numbers	OCCUPATION (Others)	Numbers
Arable	0	Meat Inspectors	1
Cowmen	25	Butchers	1
Dairy Farmers	13	Vets	2
Sheep Farmers	1	Miscellaneous	7
Beef Farmers	1		
TOTAL:	40		11

Recent epidemiological data presented in Tables III and IV shows that the new emerging hazard is associated with the *Hebdomadis* serogroup found mainly in farming and farming related occupations, and in particular, the dairy farming. The mode of infection is not entirely understood but evidence shows that modern milking parlours which place the cowman at eye level with the udders is responsible for all infections recorded at the Leptospira Reference Unit, Hereford. *(See Fig. 3).*

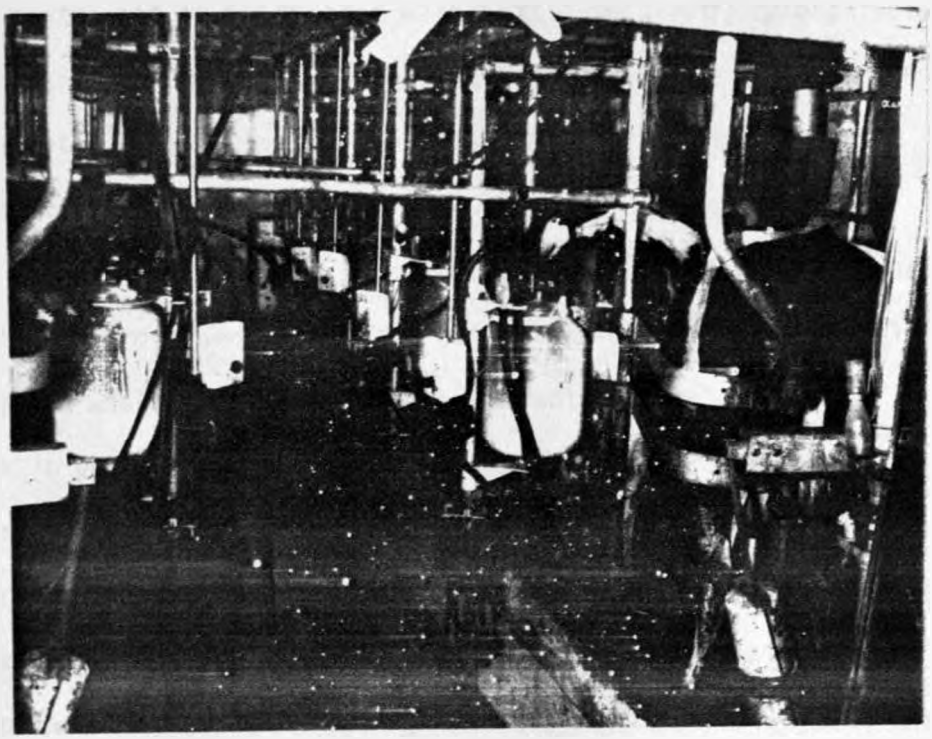
The epidemiology of leptospirosis in Great Britain, although complex, is easily differentiated into three main groups, namely: *Icterohaemorrhagiae; Canicola* and *Hebdomadis*. The major hazard today is Cattle Associated Leptospirosis in those working in the Dairy Farming Industry.

(IV) CLINICAL LEPTOSPIROSIS

Man.

Leptospirosis is not synonymous with Weil's disease, other febrile

FIGURE 3. Shows a typical herring-bone milking parlour. The cowman works at eye level with the cow's udder, and is in danger of being splashed with contaminated urine.



illnesses are also associated with leptospiral infections. These are often mistaken for Malaria, Yellow Fever, viral hepatitis and bacterial fevers in the tropics. *(Sulzer, A.J. et.al., 1976)*. In temperate climates, leptospirosis may often be diagnosed as influenza, viral hepatitis or meningitis. The severity of the disease varies from the classical characteristics of Weil's syndrome, namely: hepatorenal failure and meningitis *(Alston, J.M. and Broom, J.C. 1958)*, and occasionally in very severe cases, haemoptysis *(Fletcher, F., Johnson, R.N., Peter, S. 1976)*, and acute pancreatitis *(Kral, L. et.al., 1982)* to a milder flu-like illness with severe headache which may persist for several months and is associated with Cattle Acquired Leptospirosis.

It must be remembered that there is considerable overlap in the clinical presentation and clinical diagnosis must always be confirmed by laboratory tests.

Weil's disease is caused by *L.interrogans var Icterohaemorrhagiae*. Two distinct phases of the illness are noted, the first is essentially a leptospiraemia while the second is predominantly a leptospiruria. The bacteraemic phase corresponds to the incubation period and varies from 7-12 days; with subsequent spread to tissues and organs. This accounts for the varied presenting symptoms often noted in leptospirosis. Blood cultures taken during the first week of illness often yield positive growth of leptospira. During the second phase of illness, the leptospira have been removed from blood and tissues by phagocytes and increasing concentrations of antibodies. The kidney is the only organ which retains leptospira, these migrate to the convulated tubules where they may multiply producing renal failure. Because the kidney is involved, the leptospira may be shed via the urine and can persist for several months even after clinical cure has been achieved. However, the numbers excreted are too low to be an infectious hazard. During the

second week, increasing amounts of antibodies may be detected and by the tenth day of illness sufficient IgM has been produced and therefore, the original clinical diagnosis can be confirmed. When meningitis is a clinical feature, leptospira may be isolated from cerebral fluid and antibodies demonstrated.

Milder forms of leptospirosis occur with Cattle Associated Leptospirosis (CAL) and there is usually a history of working with cattle. The illness usually presents with flu-like symptoms, including fever, severe headaches and often mental confusion. The recovery is prolonged, usually 8 - 10 weeks and lethargy is common. In a small minority of cases the symptoms may proceed to meningitis, renal and hepatic failure and very rarely death. (See Table V)

TABLE V. Classical symptoms in man. *(Waitkins, S. 1983)*

Icterohaemorrhagiae	*Canicola*	*Hebdomadis (hardjo)*
Severe hepatorenal failure	Jaundice	Flu-like illness
Conjunctivitis	Conjunctivitis	Severe headache
Meningitis	Meningitis	Meningitis
Death	Death	

Bovine

In the bovine species, leptospirosis manifests as an acute, subacute or chronic illness. The acute phase is characterized by a fever and may be accompanied by haemoglobinuria, anorexia and jaundice. There is leptospiraemia in this phase. Calves may develop uraemia in terminal stages of the phase and die within 3 - 5 days of illness. Abortion commonly occurs in the last trimester *(Blood, D.C., and Henderson, J.A. 1963)*. However, the subacute form of the disease is the most common by far. It has a slow onset and occurs in older cattle

Milking cows decrease their lactation yield and a yellow/red thick fluid that resembles colostrum (atypical mastitis) is produced. It may also contain blood. The udders become flaccid and may be equally affected *(Mitchell and Boulanger, 1959)*. Other signs include pyrexia, decreased ruminal motility, increased pulse and respiratory rates and leukocytosis. During this phase, leptospires are excreted in milk, though milk is highly leptospirocidal and therefore of little importance in dissemination of the organisms and is not a public health hazard *(Sullivan, 1976)*.

The chronic phase of infection is insiduous and may not be detected except for persistent abortions, stillbirths, fetal death, weak calves at birth and retained afterbirths. In this phase there is leptospiruria and infected cattle may remain leptospiruric for up to 19 months, thus maintaining an endemic state in the herd *(MacKintosh, et.al., 1980a)*. Heifers that come in contact with these leptospiruric adults may become infected, thus maintaining the endemicity. This transfer of leptospires within the cows is important when heifers are housed together with adult carriers during the winter months. It is thought that housing of animals increases urine drinking and also puts stress on the animals which then shed more leptospires in large quantities in their urine.

In tropical countries, unusual clinical signs of leptospirosis were reported by Burdin, M.L., and his colleagues (1958). They studied an outbreak of leptospirosis in Kenya (due to *L.grippotyphosa*) in which pyrexia was not a constant feature but death of animals occurred within 2 - 6 days after the onset. They reported relapse in surviving cases 4 - 6 weeks after the onset of disease in which clinical symptoms were not severe, but eventually fatal. They also reported development of dysentry in some animals. Other abnormal symptoms noticed were the development of necrotic dermatitis, which was thought to be photosensitization particularly since it was most common in Ayrshire and Friesian

grade stock. The necrotic lesions on the skin were noted within 3 - 4 days of initial symptoms of the disease frequently appearing first on the teats of cows and then later spreading to other unpigmented areas of the skin. In an earlier study in Tunisia, Gayot (1955) noted the occurrence of cutaneous ulcers in acute cases of *L.grippotyphosa* in cattle. He noted that the lesions exuded a sticky fluid which coagulated on the hair, producing hard plaques. As the ulcers healed the plaques loosened and finally dropped off leaving bare patches of skin. The development of necrotic dermatitis as a result of leptospiral infection is an interesting feature and probably requires further research.

The post mortem lesions in cattle due to leptospirosis vary from animal to animal and also depend on the infecting serovar. Generally the common lesions encountered are; congestion of the kidneys with some enlargement of the whole organ. It may show light coloured patches *(Burdin, M.L., 1958)* which in later stages of the disease show as progressive interstitial nephritis manifested by small white, raised areas in the renal cortex *(Hadlow, W.J., and Stoenner, H.G., 1955)*. Burdin, (1958) noted that both the liver and spleen may show no abnormality, except for a few cases in which the liver was uniformly orange-yellow or pale. He noted that lungs were congested while the heart and abomasum showed multiple subendocardial and submucosal haemorrhages respectively.

Sheep and Goats.

The acute phase of leptospirosis in sheep and goats is characterized by snuffling, lowering of the head, some animals show jaundice and most die within 12 hours *(Burdin, M.L., 1958)*. The chronic form may occur and manifest itself by loss of body condition but abortion seems almost entirely a manifestation of the acute form *(Beamer, P.D., et.al., 1953)*. The common infecting serovars in sheep

and goats are similar to those in cattle. The most significant post mortem finding is nephritis.

Pigs.

The acute form of the disease in the pig commonly manifests as a fever with diarrhoea, convulsions and jaundice *(W.H.O., 1982)*. The subacute and chronic forms of leptospirosis are much more common. They are characterized by still birth in pregnant sows *(W.H.O., 1982)*. Abortions occur only if sows become infected during the first half of pregnancy. Infection during late pregnancy has little or no effect on litter size or health *(Blood, D.C., and Henderson, J.A., 1963)*. *L.pomona* seems to be the major leptospiral infection of the pig, though *tarassovi, australis, grippotyphosa, canicola* and *icterohaemorrhagiae* do occur and may be equally important *(W.H.O., 1982)*.

Horses.

The acute phase is characterized by fever, depression, listlessness, jaundice and at times haemoglobinuria *(W.H.O., 1982)*. The subacute form as described for cattle occurs commonly but the illness is mild and short lived *(Lyubashenko, S.Y., and Novikova, L.S., 1947, Roberts, S.J.,et.al., 1952)*. The chronic form of the disease in horses occurs and is manifested by abortions at the seventh to tenth month of pregnancy *(Jackson, R.S., et.al., 1957)* and is also associated with periodic ophthalmia. There is both experimental and serological evidence to support the long upheld theory that periodic ophthalmia (recurrent iridocyclitis) in the horse is due to leptospirosis *(Morter and others, 1964, Hathaway, S.C., Little, T.W.A., Finch, S.M., and Stevens, A.E., 1981)*. Periodic ophthalmia is a possible sequelae to infection with a variety of serotypes *(Heusser, 1952, Hanson and other, 1969, Trap and others, 1979)*. Recurrent attacks usually terminate in blindness in both eyes *(Jones, T.C., 1942)*. Serovars commonly associated with infections in the horse

include, *pomona, icterohaemorrhagiae, sejroe* and *canicola (W.H.O., 1982).*

Dogs and Cats.

In the dog, the disease due to *L.icterohaemorrhagiae* is commonly referred to as "Yellows" while that due to *L.canicola* as Canicola fever or Stuttgart disease. The *Icterohaemorrhagiae* infection has a sudden onset with high fever in the hyperacute form. Canicola fever comprises of acute vomiting, rapid dehydration and collapse. Both infections however are serious in nature and all show jaundice *(Alston, J.M., and Broom, J.C., 1958).* Haemorrhages may be present in the acute phase and may resemble *Ehlirchiosis.* Dogs rarely show the subacute form of the disease. The chronic form of the disease is characterized by nephritis which on post mortem shows as spots on the kidneys *(W.H.O., 1982).* Leptospiral infection of cats is uncommon and very rarely causes recognizable disease.

Rodents.

Rodents usually only show the acute phase of leptospirosis. The phase is characterized by fever, depression, listlessness, anorexia, conjunctivitis and anaemia. Haemorrhages and conjunctivitis may not always show *(W.H.O., 1982).* If death does not occur, then the animals become carriers for life. Rodents harbour most of the leptospiral serovars. though serovars of *Icterohaemorrhagiae, Grippotyphosa, Javanica, Hebdomadis and Tarassovi* seem to be much more common.

(V) DIAGNOSIS

Since leptospirosis may be confused with other diseases, clinical diagnosis is difficult. Definite diagnosis can only be reliably performed in the laboratory where both serological and cultural methods are employed.

Serology.

The serological diagnosis of leptospirosis is often difficult, particularly the interpretation of results. These may be masked either by "prozoning" effect of the test sera or the anticomplementary activity of the antigen used. *(Waitkins, S.A., 1983)*. However, in general, if certain ground rules are obeyed then serological diagnosis of leptospirosis can be undertaken successfully by all routine laboratories.

Despite the complex nature of the antigenic components of leptospira, antigen antibody reactions are still used in the identification of strains (as serogroups), in the diagnosis of current infections and surveys of incidence of residual antibodies (indicating past infection) in man, livestock and wild animals *(Turner, L.H., 1968a)*. The agglutinogenic properties of leptospira can be divided into groups. Studies involving adsorption of various antisera to multiple antigens have shown leptospires to possess many agglutinogenic factors (at least 250 are already known).

There are three established serological methods used in Great Britain: the Macroscopic (slide) Agglutination Test, which is a rapid indicator of the presence of leptospiral antibodies; the Microscopic Agglutination Test (MAT), which is a serogroup specific confirmatory test and the Complement Fixation Test (CFT), which is a genus specific test and can be used routinely as a screening test. In addition the Enzyme Linked Immunosorbent Assay (ELISA) is currently under evaluation.

Macroscopic (slide) Agglutination Test.

This is a simple slide agglutination method similar to those carried out for β haemolytic *Streptococci, Salmonella* and *Shigella*. The antigen is a boiled and washed suspension of *L.biflexa var Semaranga serovar patoc* in azide saline 5 X 10^9 leptospires/ml. This antigen is genus specific

and will react with all leptospira. The test is carried out using a neat and 1:10 dilution of the patient's serum. The dilution 1:10 helps to overcome prozoning effect which may be misinterpreted as a weakly positive result. One drop of antigen and sera are mixed on a glass slide with an applicator then rotated at 125 rpm for 4 minutes. Positive and negative serum controls are included. A strong agglutination compared with controls indicates the presence of leptospiral antibodies *(See Figs. 4a and b)*.

The macroscopic slide agglutination test is less sensitive than the other tests described and it can easily be misinterpreted, on the other hand, it is a rapid indication of leptospiral antibodies. It becomes weakly positive towards the end of the first week of illness and persists for several months after recovery.

Microscopic Agglutination Test (MAT)

The microscopic agglutination test (MAT) may be performed using either live leptospires as antigen or formalised cultures, and is serogroup specific.

Live antigen; this is a 4 - 10 day old fluid culture of leptospires incubated at $30^{o}C$ and the density is approximately 1×10^{8} organisms/ml. It is important that the culture should be free of aggregates of organisms, these tend to make reading of the test difficult. Alternatively, formalised cultures of leptospires may be used. The cultures are grown as described above and killed with 0.2% vol/vol neutralised formaldehyde. In both cases the test is performed using a battery of leptospiral antigens representing all known serogroups as in Table I.

The patient's serum is initially screened at a dilution of 1:20, one drop of diluted serum is placed into either a 80 well W.H.O. plate

FIGURE 4a. Illustrates a positive Macroscopic (slide) Agglutination reaction. Note the clumping of the antigen with a cleared background, this is considered to be a STRONG POSITIVE reaction usually scored as +4.

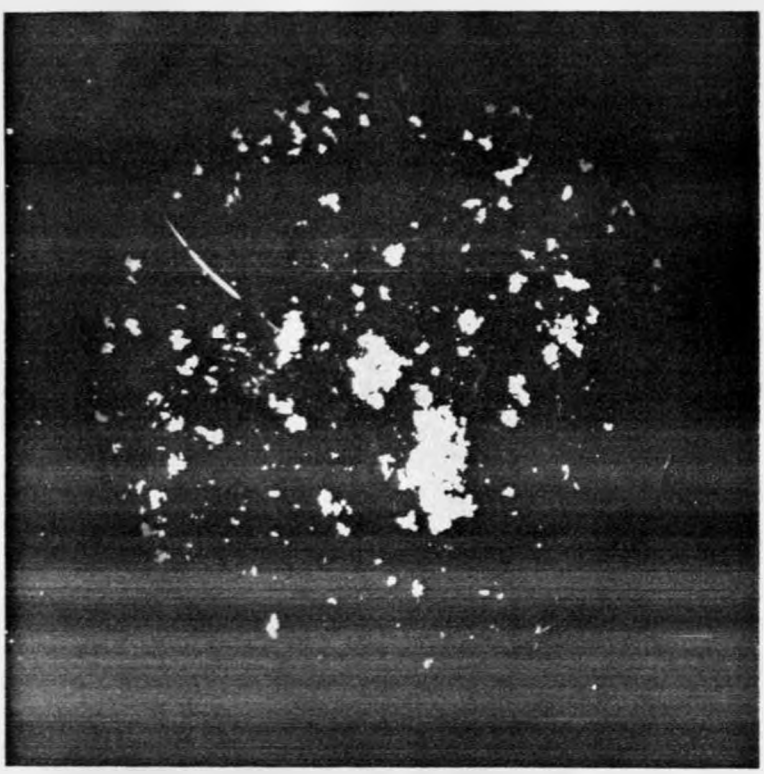
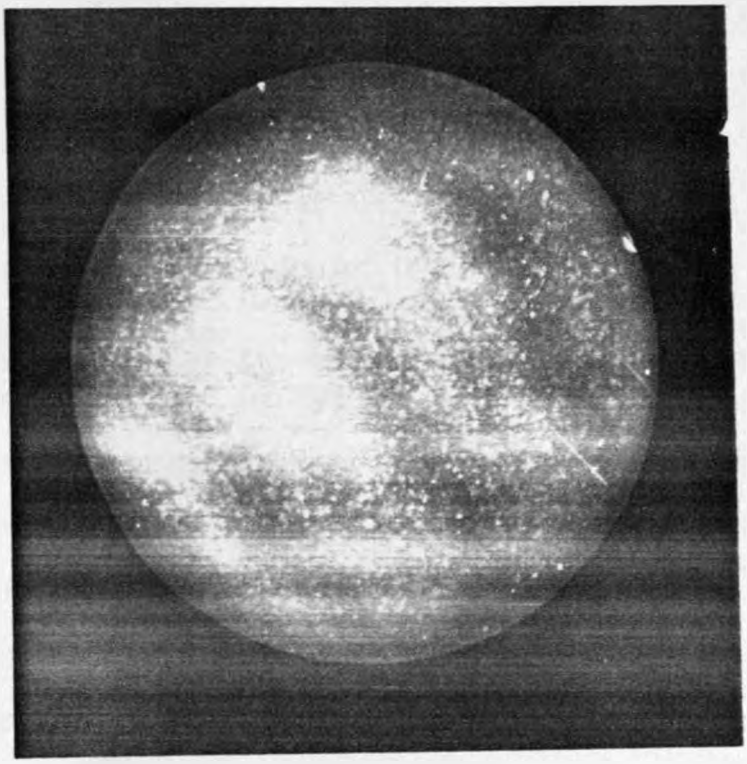


FIGURE 4b. Illustrates a negative Macroscopic (slide) Agglutination reaction. Note the absence of clumping and the uniform suspension of the antigen which is demonstrated by a grey background.



or a microtitre tray, a similar volume of serogroup antigen is added making a final dilution of 1:40. Further dilutions to 1:5200 are undertaken if the macroscopic slide is positive. If live antigens are used then these are incubated at 37°C for 2 - 4 hours, if formalised, then incubation is overnight at room temperature. After incubation a drop of the antigen-antibody mixture is placed on a slide and viewed by dark ground microscopy with a X 40 objective and X 10 ocular lenses. When live cultures are used it is essential that care is taken to avoid contact with the drop and the slide must be discarded immediately into disinfectant. In living suspensions the network of live leptospires form compact, bright white balls indicating that the antibody present has agglutinated the leptospires. If no antibody is available, then no agglutination takes place *(Figs. 5a, b & c)*. When formalised antigens are used the clumps tend to be larger and less tightly packed with irregular outlines giving a more "lacey" appearance. Dense balls of leptospires do not occur. An agglutination is positive if 50% of leptospires are agglutinated,, the end point is the lowest dilution of antibody showing 50% agglutination *(Figs. 6a, b & c)*.

The MAT detects both IgG and IgM and may initially cross-react with several leptospiral serogroups. However, as the patient recovers the infecting serogroup will eventually predominate and this may take several weeks and sometimes even months. Borg-Peterson (1949) noted that in the first few weeks of disease it was not uncommon that heterologous reactions were stronger than homologous reactions. This is what is referred to as the "paradoxical reaction" by Fuhr in 1950. Kmety in 1958 noted that the paradoxical reactions occur mostly between 6 and 11 days from onset of illness in about 50% of cases, and are given by some strains (but not all) of various serogroups. He noted that these reactions were most frequent in infections due to *Icterohaemorrhagiae* and

FIGURE 5a. Microscopic Agglutination Test using a LIVE leptospiral culture as antigen. The resulting agglutination appears as a compact, bright white ball and is scored +, indicating 100% agglutination.

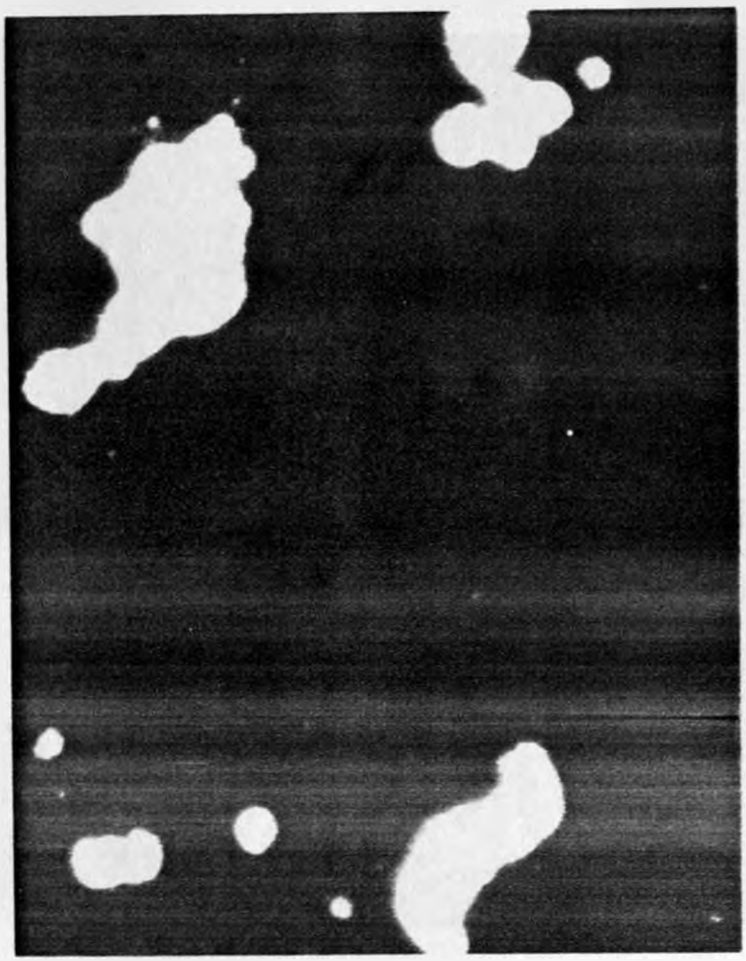


FIGURE 5b. Illustrates the same Microscopic (slide) Agglutination Test using LIVE antigen. This is a negative reaction and shows free leptospiral organisms.

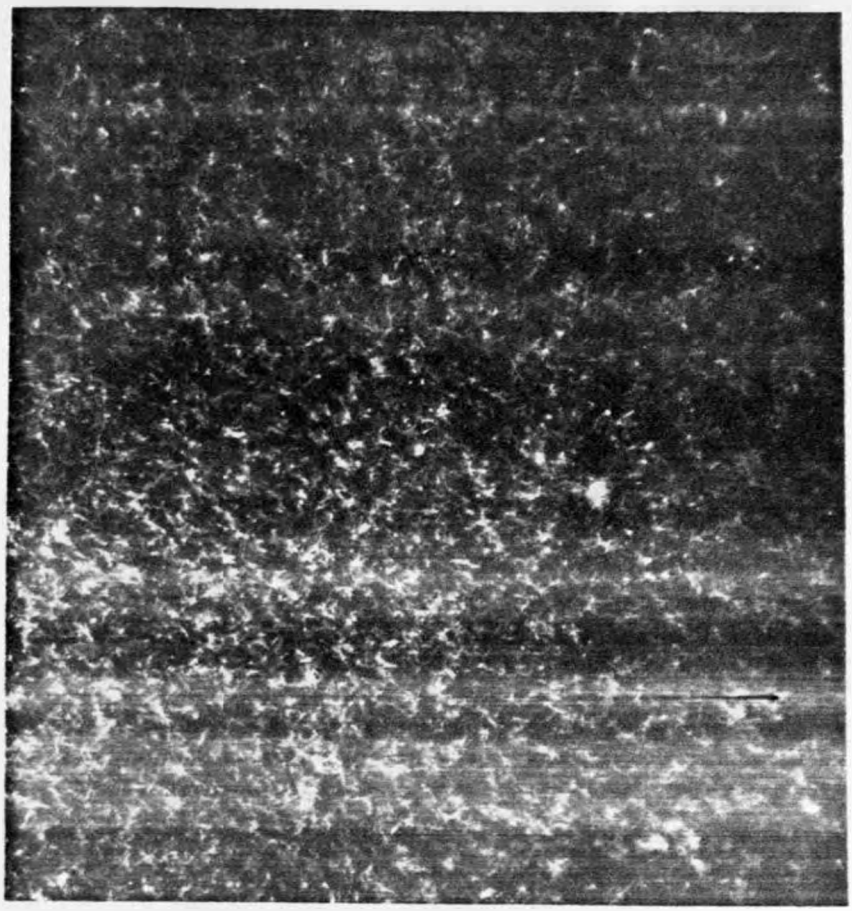


FIGURE 5c. Illustrates a 50% [+/-] agglutination in the LIVE Microscopic Agglutination Test. This indicates the end point of the serological reaction.

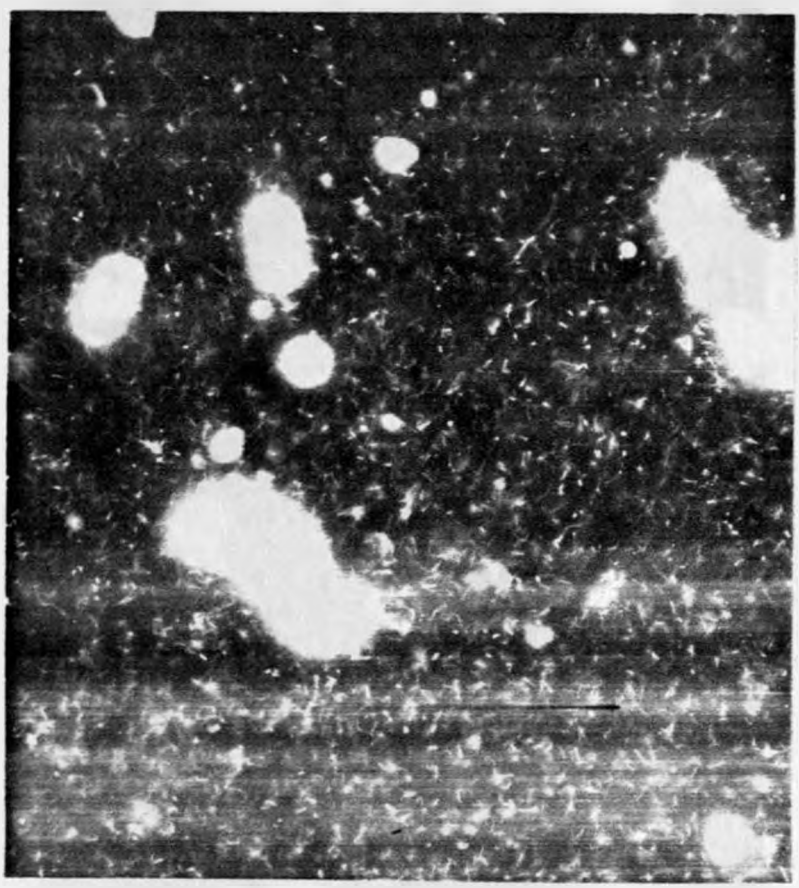


FIGURE 6a. Microscopic Agglutination Test using formalised leptospires. 100% agglutination is shown. Note that in Figures 6a & 6b the agglutination appears as "lacey" antigen-antibody complex when compared to Figures 5a & 5c.

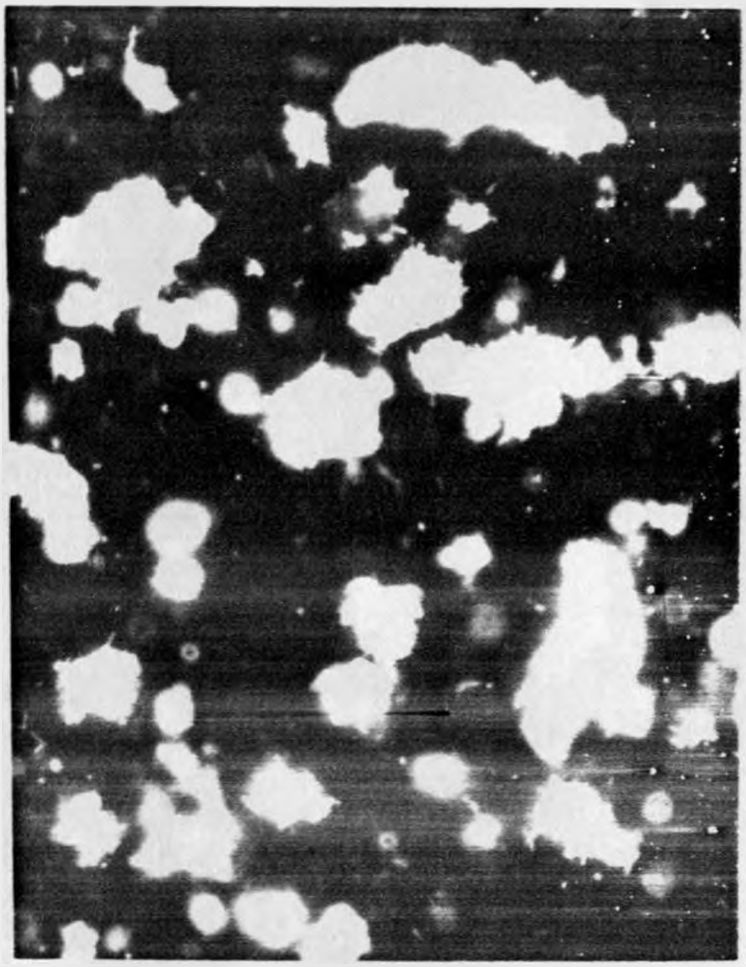
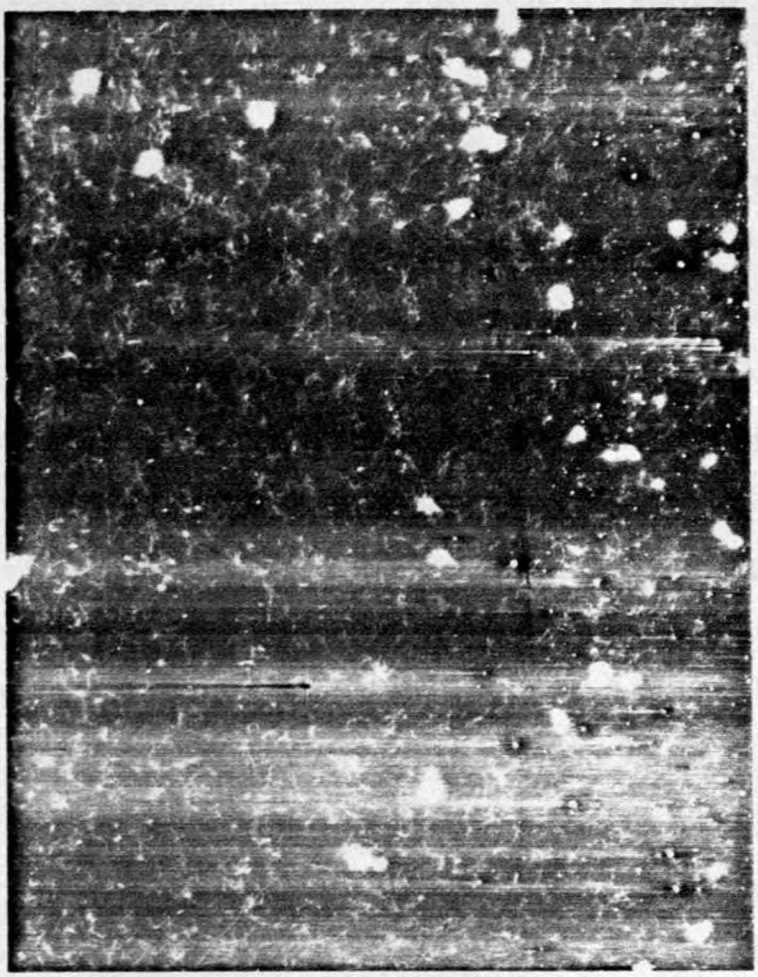


FIGURE 6b. Microscopic Agglutination Test using formalised leptospires. 50% agglutination is shown and indicates the end point of the serological reaction.

FIGURE 6c. Microscopic Agglutination Test using formalised leptospires. No agglutination is noted and free leptospires can be seen. This is a NEGATIVE reaction.



Pomona. The significance of the paradoxical reactions is not clear. However, if sera react to high titres in this fashion, agglutination-absorption studies will indicate the probable serogroup of the infecting serotype *(Turner, L.H., 1968)*.

Results of agglutination tests should be cautiously interpretated as "potentially serogroup indicative" *(Turner, L.H., 1968)*. This is based on three sets of factors: *(1)* The antigenic complexity of leptospires which is demonstrated by cross agglutination test. *(2)* The quality of antigen suspensions which can affect the titres. *(3)* Possibility of double (? multiple) infection with strains belonging to different serogroups which may result in confusing patterns of cross reactions.

Complement Fixation Test.

A compound screening antigen is used in the leptospiral CFT. This comprises organisms from the serogroups, *Patoc, Canicola, Hebdomadis* and *Autumnalis* and then acid and heat treated for use.

Two particular points must be noted. Incubation of the leptospiral CFT must be prolonged at $4^{o}C$ overnight and it is important to remember that the above antigenic preparation is slightly anticomplementary and therefore, the complement used in the test proper should be at a strength of $2MHD_{50}$ and not $3MHD_{50}$ as commonly used in virology screening. Otherwise the leptospiral CFT is similar to that used in routine virology testing, a positive control antisera should always be included. If the above method is followed then routine screening for leptospirosis, particularly from the farming community, may be undertaken with confidence by all laboratories. Any suspect positive results must be confirmed by the Reference Laboratory because occasionally the CFT antigen cross-reacts non-specifically with Hepatitis A, Cytomegalovirus and Mycoplasma antibodies. CFT antibodies are usually detected about 10 days after the

date of onset but may be delayed for as long as 21 days. Therefore, it is essential if leptospirosis is suspected clinically that a second sample of serum should be tested to confirm the original diagnosis. There have been incidents where true cases of leptospirosis have been disregarded because the initial CFT was negative *(Waitkins, S.A., 1983)*.

The CFT is useful to routine laboratories only if the laboratory methods outlined above are followed. However, changing complement strength and incubation times are inconvenient in a busy laboratory, therefore, alternative screening methods must be developed. Such a test is the Enzyme Linked Immunosorbent Assay (ELISA).

Enzyme Linked Immunosorbent Assay (ELISA).

The ELISA test is currently being developed for diagnosis of leptospirosis in both man and animals. The transient nature of the immunoglobulin M (IgM) response as measured by ELISA indicates potential usefulness as a serodiagnostic test for detecting current leptospiral infections *(Adler, B., Cousins, D.V., Faine, S., and Robertson, G.M., 1982)*. The antibody response in both humans and animals to leptospires follows the classical IgM and IgG response pattern *(Graves, S.R., and Faine, S., 1970)*. ELISA techniques have thus been developed to detect specific anti-leptospiral immunoglobulins M and G in human sera *(Adler, B., Murphy, S.M., Locarnini, S.A., and Faine, S., 1980, Terpstra, et.al.,1980)* and in bovine *(Adler, B., Cousins, D.V., Faine, S., and Robertson, G.M., 1982)*. The detection of specific IgM by ELISA appears to be suitable as a diagnostic test, particularly as many laboratories are now using this technique for serodiagnosis of infections and it would be simple to include leptospira-coated plates in a battery of tests. Moreover, leptospira antigens adsorb easily on plastic microtitre plates and the antigen coated plates are stable at -20^{0}C for several months *(Biancifiori, and Cardacas, P., 1983)*. ELISA can easily be automated, the results

obtained by visual and spectrophotometry do not vary greatly *(Adler, B., Murphy, A.M., Locornini, S.A., and Faine, S., 1980, Terpstra et.al., 1980)* thus enabling the test to be of use in routine laboratories.

The method used in the Reference Laboratory is that developed by Terpstra and his colleagues in Amsterdam *(Terpstra, et.al. 1980)*. The antigen preparation is a single serogroup grown for 4 - 7 days in EMJH media at 30°C in a shaking incubator to a density of approximately 11 X 10^8 organisms/ml. The culture is killed by formalin (final concentration 0.5%) and then heated at 100°C for 30 minutes. After cooling the killed culture is centrifuged for 30 minutes at 1600 g. The supernatant is used as the antigen. 100µl of this preparation is dispensed into each well of microtitre plates and left at room temperature for 2 - 4 days until excess fluid has evaporated leaving a concentrated antigen fixed to the plate well. The plates can be stored and used as necessary. At the Reference Laboratory, the ELISA method performed uses anti-human peroxidase conjugated immunoglobulin (Institut Pasteur) with 5 amino salicylic acid (Merck) as substrate.

The antibody levels detected by ELISA when compared to MAT do not correlate. It is probable that the two techniques measure different antibody systems *(Bianchfiori, F., and Cardacas, P., 1983)*. ELISA has a higher sensitivity and precociousness than MAT, and it offers the advantage of handling a non-infectious antigen. ELISA permits the test of large number of sera in a few hours and results can be available the same day. Initial results comparing the ELISA method with MAT and CFT indicate that it could easily be adapted for routine use.

Others.

Other serological tests employed in the diagnosis of leptospirosis include sensitized erythrolysis (SEL) and immunofluorescent antibody technique (IFT). The fluorescent antibody technique (FAT) is a very useful method especially in veterinary medicine for the diagnosis of leptospirosis from aborted fetus. Normally, after fetal death, there follows autolysis which kills the leptospires and renders them uncultivable *(Ellis and Michna, 1976)*. A fluorescent antibody technique (either direct or indirect) may be of value in establishing leptospiral infection where abortion has occurred and has taken time before samples are submitted for culture.

Cultural methods.

The success and reliability of laboratory investigations depend largely on specimens of good quality and adequate amounts obtained at the right time in the course of infection. Samples from both man and animals for culture include urine from the leptospiruric phase (after 10 days) and blood in the acute phase (the first 10 days). Cerebrospinal fluid may be of value in the leptospiruric phase for culture.

Some factors which affect the viability of leptospires *(Turner, L.H., 1970)* include: (i) Imperfectly cleansed glassware that may contain traces of detergent, (ii) unsuitable media; normally rabbit serum (pre-tested for the absence of leptospiral antibodies) is used in media. The pH range should be 7.2 - 7.4, (iii) inhibitory substances in the inoculum such as antibodies and some lipids. The inocula should therefore be small in volume or diluted in medium for better isolation *(Menges, W., Rosenquist, D., Galton, M., 1960, W.H.O., 1967, Hathaway, S.C., Little, T.W.A., and Stevens, 1982a,b)*, (iv) antibiotic therapy reduces the chances of isolation, and (v) contamination with

other microorganisms. These tend to reduce the chances of isolating leptospires.

Various formulae of media are in use today for isolation and cultivation of leptospira and include Fletcher's (1928); Korthof's (1932) or modifications of it *(Alston and Broom, 1958, Babudieri, 1961)* Stuart's (1946) and modifications of it *(Ellinghausen, 1969)* and Vervoort's modified by Wolff (1954). The most commonly used media in the laboratory however are: Ellinghausen and McCullough (1965), Ellinghausen and McCullough modified by Johnson and Harris - EMJH - (1967) or EMJH with 5-Fluorouracil - (5-FU) *(Johnson, R.C., and Rogers, P., 1964)*.

5-Fluorouracil (5-FU) was found to be lethal to most microorganisms but surprisingly not to leptospira at concentrations of 200-400 µg/ml by Johnson and Rogers (1964). Johnson and Rogers found out that pyrimidines were not incorporated into the nucleic acids of leptospira while purines were. 5-FU is a pyrimidine and is incorporated into the nucleic acids of other microorganisms producing lethal effects. EMJH media with 5-FU is therefore widely used in the isolation of leptospira organisms especially from contaminated samples *(W.H.O., 1967, Hathaway, S.C., Little, T.W.A., Stevens, A.D., 1982)*. EMJH with 5-FU media has proved useful in epidemiological surveys for leptospira.

Addition of agar at 0.1 - 0.2% level enhances the isolation rate. Agar either removes inhibitory factors or adds to the nutritive requirements or both *(Cerva, 1967)*. Samples from contaminated specimens such as urine give positive results more readily if they are serially diluted. *(Menges, W., Rosenquist, D., Galton, M., 1960)*. Serial dilution reduces the inhibitory substances such as lipids, antibodies and other bacteria thus increasing the chance of isolation.

The optimal incubation temperature for cultivation of of leptospira

is 28 - 30°C. Samples may be discarded as negative after 60 days if growth is not observed. In some rare instances, samples that may appear negative are found to be positive on blind passage. Obviously such specialised media as mentioned above cannot be easily stocked in routine laboratories. Alternative procedures for the successful isolation of the leptospires must be used. Palmer and his colleagues found that certain commercial blood cultures supported the survival of leptospira better than others *(see Table VI)*. Those media that provided good nutritive source, had adequate aerobic conditions, and were incubated at 30°C enabled *L.interrogans* to survive up to 9 weeks. Daily and weekly microscopic examination of blood culture systems is required. It is therefore recommended that for routine isolation of the organism it is probably better, after consultation, to refer such suspect cultures to the Leptospira Reference Laboratory.

TABLE VI. Survival times of 3 wild strains of *L.interrogans* at 37°C and *L.interrogans var Wolffi serovar hardjo* at 30°C in commercial blood cultures. *(Palmer, et.al. 1983)*

Supplier	*Ictero*	*Canicola* 37°C	*Hardjo*	*Hardjo* 30°C
OXOID	-	48 hrs	48 hrs	1 week
DIFCO (CO_2vac)	-	-	-	-
DIFCO (CO_2N_2)	-	-	48 hrs	48 hrs
GIBCO	43 hrs	48 hrs	48 hrs	48 hrs
LAB M	1 week	2 weeks	2 weeks	7 weeks
BACTEC (O_2)	1 week	1 week	1 week	7 weeks

(VI) CLINICAL MANAGEMENT

Man

The primary aim in clinical management of leptospirosis is to prevent the organism from invading tissues, particularly the kidneys. The principles of treatment are the same irrespective of the infecting serogroup, namely early adminstration of antibiotics, maintenance of water and electrolyte balance and supporting renal and cardiac function.

In man the place of antibiotic treatment for classical Weil's syndrome caused by the serogroup *Icterohaemorrhagiae* is still contentious. High doses of penicillin at least 8 - 10 mega unit per day, should be given within 4 days of the start of fever; if the patient is seriously ill then higher doses of 20 mega units per day have been recommended *(Turner, 1967)*. On the other hand, studies by Hall and his colleagues in 1951 and Fairbain and Semple, 1956, demonstrated that there was no benefit to be gained from using penicillin treatment. Despite the uncertainty, and until there is strong medical evidence against treatment, clinicians should be receommended to start parenteral penicillin as soon as possible. Alternatively, if the patient is hypersensitive to penicillin then tetracycline at initial dose of 500 mg, followed by 250 mg 8 hourly intravenously for 24 hours and then 500 mg 6 hourly orally for 10 days may be used, or erythromycin given 250 mg 6 hourly for 10 days.

The antibiotic treatment of cattle associated leptospirosis (caused by *L.hardjo*) is less controversial. Oral amoxycillin given at 500 mg 8 hourly for at least 5 days has proved to be effective, Treatment may be started at any stage of the illness and recovery is quick, usually within 24 hours of administering the antibiotics. Patients with mild infections usually recover without antibiotic treatment but their symptoms may be prolonged for several months.

Animals

In animals, the establishment of renal tubular infection, coinciding

with the onset of leptospiruria may persist for many months. In domestic animals this will act as the means of transmission within a herd, and dihydrostreptomycin is the drug of choice *(Ringer, et.al., 1955, Doherty, 1967, Stalheim, 1967, 1969)*. The same drug is useful in humans during renal involvement.

In the horse where leptospirosis is thought to be the cause of periodic ophthalmia, steroid therapy may be useful in the treatment of the condition *(Blood, D.C., and Henderson J.A., 1963)*, in addition to the antibiotics.

(VII) CONTROL

Leptospirosis is a notifiable disease in most countries and guidelines for it's control have been drawn up by the World Health Organization *(W.H.O., 1982)*. In man, prophylactic measures against leptospirosis include personal hygiene and care, environmental hygiene and abortive treatment *(W.H.O., 1982)*. The traditional way of protecting milkers from contact with infected urine appear ineffective and possibly the best way to achieve prevention of leptospirosis is by elimination of infection from cattle, especially by their vaccination *(MacKintosh, et.al., 1982, Ryan, T.J., Hellstrom, J.S., and Pinniket, J.H., 1982, Hathaway, S.C., 1981)*. There is no evidence however, that vaccination affects the intensity and duration of leptospiruria in animals infected at the time of vaccination *(Hubbert, W.T., and Miller, J.N., 1965, Babudieri, B., et.al., 1973)*. Vaccination should be instituted before active infections are established (i.e. during the months of low incidence). Gamma-irradiated vaccines have proved effective in both protecting animals from disease and from infection to become carriers. *(Hubbert and Miller, 1965, Stalheim, 1967, 1968)*. Control of leptospirosis in domestic animals depends on lowering the

prevalence of infection with serovars maintained within populations of domestic animals and decreasing the degree of ecological association with free living maintenance host populations. On the farm, the pig is the major maintenance host for *Pomona* while the bovine is the major maintenance host of *hardjo (S.C. Hathaway, 1981, MacKintosh, et.al., 1982)*. Vaccination of pigs against *Pomona* and cattle against *hardjo* will considerably reduce the prevalence of infection in vaccinated and therefore decrease the probability of accidental *Pomona* and *hardjo* infections in man *(Ryan, T.J., Hellstrom, J.S., and Penniket, J.H., 1982)*. It is recommended that new livestock to be introduced on a farm should be treated with dihydrostreptomycin to eliminate the renal infection *(Ryan, T.J., Hellstrom, J.S. and Penniket, J.H., 1982)*. It is always worthwhile to determine the history of any new animal before it is purchased for the purpose of introducing it to the farm.

Recommendations for prevention of leptospirosis by the World Health Organization are laid down *(W.H.O., 1982)*. These are as follows:

(A) Control of infection in man

(i) Rodent control in areas of food production

(ii) Rodent control in domestic environments.

(iii) Control of exposure in an infected environment:

- (a) Urine
- (b) Animal tissues
- (c) Water and mud

(iv) Occupational hygiene.

(v) Immunization

(B) Control of infection in animals

(i) Rodent control:

- (a) Poisoning
- (b) Use of predators
- (c) Other methods.

(ii) Chemotherapy for domestic animals. Usually penicillin streptomycin combination is sufficient.

(iii) Isolation of healthy from the sick, (quarantine).

(iv) Slaughter of the sick domestic animals

(v) Ecological control of feral population

(C) General control measures

(i) Chemical treatment of water, soil and ricefields.

(ii) Occupational hygiene.

In carrying out a successful control programme, epidemiological investigations are very important and these include:-

(i) Identification of cases; both clinically and laboratory confirmation.

(ii) Reporting and collection of surveillance data.

(iii) Search for the source of individual infections and epidemic and epizootic waves.

(iv) Based on the above, establish the level of endemic or enzootic infection.

(v) Advice on the implementation of control action.

Since leptospirosis is a classical zoonosis, educational programmes must involve; veterinary, medical, public health and wild life control programme personnel. Farmers, meat handlers and others with direct contact with animals have to be warned of the potential dangers of contracting leptospirosis if personal hygiene measures are ignored. In carrying out control programmes, it is essential to have the cooperation of the administrative machinery from the lowest level up to the national level.

(VIII) CONCLUSION

In conclusion, leptospirosis is a zoonotic disease. The main reservoir of infection are rodents and other domestic animals, such as dogs and cows. Man becomes infected through direct or indirect contact

with contaminated urine. The economic implications of infection can be quite serious.

The laboratory diagnosis of leptospirosis depends on the detailed information of high risk factors, e.g., is the patient a farmer? have his cows mastitis? does the patient pursue water sports? does the patient have any relevant clinical signs? Awareness of the epidemiology and clinical symptoms of the patient may lead to suspicion of the disease. Actual diagnosis of leptospirosis relies mainly on serological evidence of infection, complement fixation test may be used as a routine screen and positive findings confirmed by the Reference Laboratory. In future the ELISA will hopefully replace the CFT and provide not only a genus but serogroup specific result.

INTRODUCTION TO THESIS

The first wild animals found infected with leptospires were rats and these were considered as an important source of infection for man *(Noguchi, 1917)*. It was later found that dogs, pigs and cattle also had leptospirosis and these also infected man. It is now known that leptospirosis is widespread among domestic and wild animals in the world *(Galton, M.M., 1959a, b and Appendix I)*, that it is caused by different leptospires and is probably the world's most widespread zoonosis *(Van der Hoeden, 1964)*.

The knowledge of the wide distribution of leptospires in such a variety of wild animals throughout the world is of great importance to public health workers whose role it is to inform the general community of the health hazards. The public health worker is therefore charged with the responsibility of carrying out surveillance programmes to locate carrier animals for zoonotic diseases, including leptospirosis. Carrier animals in any given locality represent both predictable and unpredictable sources of leptospiral infection for man and domestic animals. It is known that certain leptospiral serotypes have a primary animal host though they may infect other animals as well. The organisms reside in the kidney and length of shedding depends upon the host-serotype relationship. The degree of shedding is not known in many host-serotype relationships, especially in the wild life species. It is therefore important to regard any animal as a potential source for infection.

Because of the wide range of clinical manifestation of leptospirosis, the diagnosis of the disease cannot be made on clinical signs and symptoms alone. Reliable diagnosis can be achieved by a combination of good history taking, clinical examination, cultural and serological investigation.

Serological tests for leptospirosis are used for the diagnosis of cases in both man and in domestic animals. Some tests are used for epidemiological investigations and frequently entail retrospective determination of antibodies which may have been evoked many months previously. The microscopic agglutination test, when used with a good selection of

antigens, may be used in this way. However, prevalences of leptospirosis based on serological studies alone are questionable because; (1) serological data are not sufficient to tell with certainity the infecting serotype, (2) infections may be present to serotypes as yet unknown, (3) leptospires may be present in the absence of serum antibodies and (4) heterologous titres may equal homologous titres. Prevalences based on bacteriological examination is preferred because (1) the objections of serological studies are overcome, and (2) the leptospire isolated can be identified *(Roth, et.al., 1961a)*. Prevalence studies based on both serological and becteriological methods yield the most information. Such studies may not only reveal additional leptospiral serotypes, host species and reservoirs in any given locality, but provide valuable information regarding the ecology of the disease.

The identification of the infecting serovar is obviously important for epidemiological investigations, but it has little or no importance for the clinician or veterinarian in the management and treatment of a case. Consequently, a laboratory diagnosis of leptospirosis serves the primary needs of the clinician or veterinarian. Currently, the slide agglutination test (SAT) is used for this purpose.

Though the SAT is a rapid screen test for leptospirosis, there still exists differences in the way different laboratories and even individuals within the same laboratory read results. This discrepancy is probably due to the wide range of scores (+4 to -) within which an individual may place his/her results; thus making it possible to group slight positive reactions as trace and subsequently interpreting them as negative for the purpose of reporting. There is, therefore, the need to remove these borderline case results by improvement of the test. This calls for either the amplification of the results such that a precise positive is easily differentiated from a negative with little doubt, or the use of stained antigens for the easy reading by some people. The use of stained antigen has already been investigated by Bragger, J.M. and Adler, B. *(1976)*. It is important to realise

that a quick, simple and precise test is the ultimate need for any diagnostic laboratory, especially so in countries where trained manpower is scarce.

The aims of this project are:

(i) To carry out a short bacteriological, serological and histological study of the evidence of leptospirosis in the Coypu (*Myocastor coypus* Molina)

(ii) To carry out a short comparison study of the commercially produced slide agglutination test antigens with that produced at the Leptospira Reference Laboratory, Hereford, in the screening and diagnosis of leptospirosis.

Therefore, the project has been divided into three sections, Section A deals with the studies of leptospirosis in the Coypu (*Mycocastor coypus* Molina) while section B deals with the comparison of the slide antigens. Section C deals with the discussion of the project as a whole and leptospirosis in general.

SECTION A.

CONTENTS

INTRODUCTION OT SECTION A.

The Coypu *(Myocastor coypus* Molina) is an aquatic rodent, native of South America which was exported to Belgium, France, Great Britain, Holland, Israel, Italy, Japan, Kenya, Poland, Turkey, U.S.A., U.S.S.R., and West Germany for the purpose of fur farming. It was first introduced in Great Britain in 1929 but later escaped into the wild (*see Figure 7a & b).* In this country, the Coypu is classified as a pest because of it's destructive activities on crops; mainly root crops, and on dykes and river banks.

It has been recognized that wild animals can play an important part in the maintenance and spread of diseases among the domestic species and man. The danger of transmission of diseases from wild life to man and domestic animals is most in countries where the wild-life reservoir is dense or in small densely populated areas, or islands where direct or indirect contact with even small numbers of wild animals can be even greater. In Great Britain, leptospires from at least seven serogroups have been isolated from wild animals, i.e., *Icterohaemorrhagiae* from rats *(Rattus norvegicus)* by Middleton *(1929),* Broom and Gibson*(1953)* and Broom *(1958); Ballum* from field mice *(Apodemus sylvaticus*), short tailed vole *(Microtus agrestis)* and bank vole *(Clethrionomys glareolus);* and *Hebdomadis* from short tailed voles and bank voles *(Broom and Coghlan, 1958); Australis serotype bratislava* from a hedgehog *(Erinaceus europaeus europaeus)* by Broom and Coghlan *(1960) Autumnalis* (serotype *erinacei auriti*) from a water vole *(Arvicola amphibus)* by Slavin, et.al., *(1964); Javanica* from hedgehogs *(Turner, 1969)* and a *Pomona* strain from a field vole *(Little and Salt, 1975., Salt and Little, 1977).*

Coypus are thought to be hosts of several bacterial, viral, fungal and parasitic diseases that are of public health importance *(M.A.F.F.,1978).* Reports on the presence of leptospires in the coypu have appeared in

FIGURE 7a. Shows a Coypu (*Myocastor coypus* Molina)

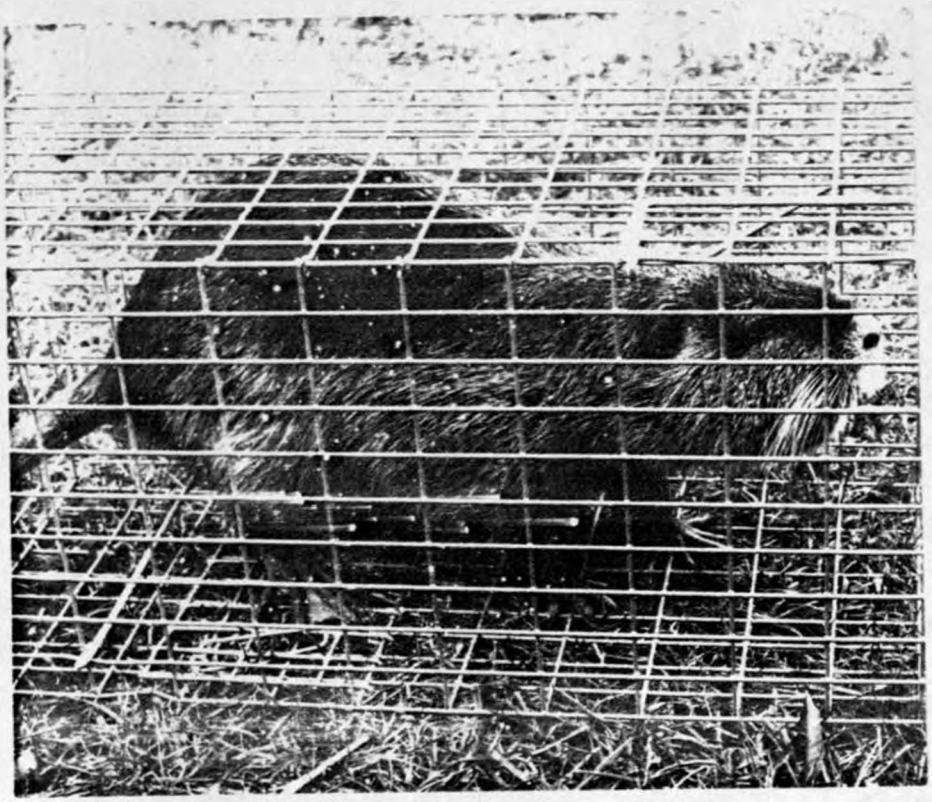


FIGURE 7b. Shows the map of the distribution of coypu in England and Wales. The coypu infested areas are marked with black etchings *(see Figure 8)*

literature. The first report was by Ancherzar and colleagues in 1949 from Argentina. They isolated a strain which they erroneously designated as *L.bonariensis* but later found to be *L.icterohaemorrhagiae*. Roth, et.al., *(1961a)*, indicate that the coypu harbours leptospires and indeed isolated six strains, and in 1962 typed the isolates as *L.bataviae* serovars- *paidjan, djatzi* and *bataviae, L.australis* and one unidentified. In 1963, Roth, et.al., isolated *L.pyrogenes serovar myocastoris* from a coypu. In U.S.S.R., Dzabrailova *(1969)* isolated *L.australis australis* from the coypu. In England, Twigg and Cuerden *(1966)* made a study of leptospirosis in British mammals and were only able to demonstrate the presence of leptospiral antibodies in the coypu they were unable to isolate the organism. In their study of the distribution of leptospires in the kidney tubules of some British mammals, Twigg and Pox *(1976)* made no study of the coypu.

The aims of this project are therefore to:

(i) Demonstrate the presence of leptospires in the coypu by culture using both Ellinghausen McCullough (EM) media with 2% rabbit serum, 0.1% agar and 0.02%/ml 5-Fluorouracil (5-FU) and EM with 2% rabbit serum, 0.1% agar but without 5-FU.

(ii) Demonstrate the serological incidences of leptospirosis in the coypu by Slide Agglutination Test (SAT), Complement Fixation Test (CFT) and Microscopic Agglutination Test (MAT)

(iii) Demonstrate histological evidence of leptospirosis in the coypu by silver impregnation technique (Warthin-Faulkner).

(II) MATERIALS AND METHODS TO PROJECT 1.

The materials used in the project and technical procedures for preparation of media are described in appendix II.

1. FIELD METHODS

(i) Area where the project was carried out.

The coypus were collected from nine parishes in Norfolk and Suffolk. Three coypus were collected from Burgh St. Peter's parish on Mr. Bryan's farm on the banks of the river Waveney, four from Barnby parish on dykes, one from Bantely parish on a stream (Dodnash brook), two from North Cove parish on Mr. Blower's farm, one from Tattingstone parish on Mr. Pluck's trout lake, two from Somerleyton parish and one from Herring Fleet Road, both on the banks of the river Waveney, five from Fritton on the banks of river Waveney and eleven from Haddiscoe ("the island") on the banks of river Waveney *(see Fig. 8 and Appendix III)*. The terrain of the land in which the coypus were caught was agricultural land used as pasture for cattle as well as for crop farming. It was interspersed with marsh *(see Fig. 9)*.

(ii) Methods of trapping and collection of specimens.

A total of 29 coypus were caught using cage traps set on their runs *(see Fig. 10)*. Either carrots or apples were used as bait. The traps were checked one day after setting them and the coypus caught and killed with a .22 pistol by shooting in the head just above the centre of the eye level while still in the cage. Blood was collected immediately from the bullet wound into 10ml plastic tubes with beads coated with kaolin as coypu blood clots very fast by the time the animal is opened up. The tubes were shaken well enough to let the beads aid fibrination *(see Fig. 11)*.

The animals were then laid on a 1 X 0.5 M hard board dorsoventrally and wetted with water and disinfected with surgical spirit. There were asceptically opened up ventrally and by use of a 5 ml syringe and 19 guage

FIGURE 8. Shows a relief map of the detailed distribution of coypu in East Anglia.
There are two overlays.

Overlay 8a. Shows the boundaries of the different densities of the coypu population. The most heavily populated area is that outlined in C1 with decreasing numbers found in C2, B1, B2, B3, and the least populated areas are found in P1 and P2 decreasing westwards.

The area MY is a designated research area controlled by the Coypu Research Laboratory, Norwich. It is used to study the ecology and methods of trapping coypu.

Overlay 8b. Shows the sites where coypu examined in this study were trapped.

FIGURE 8

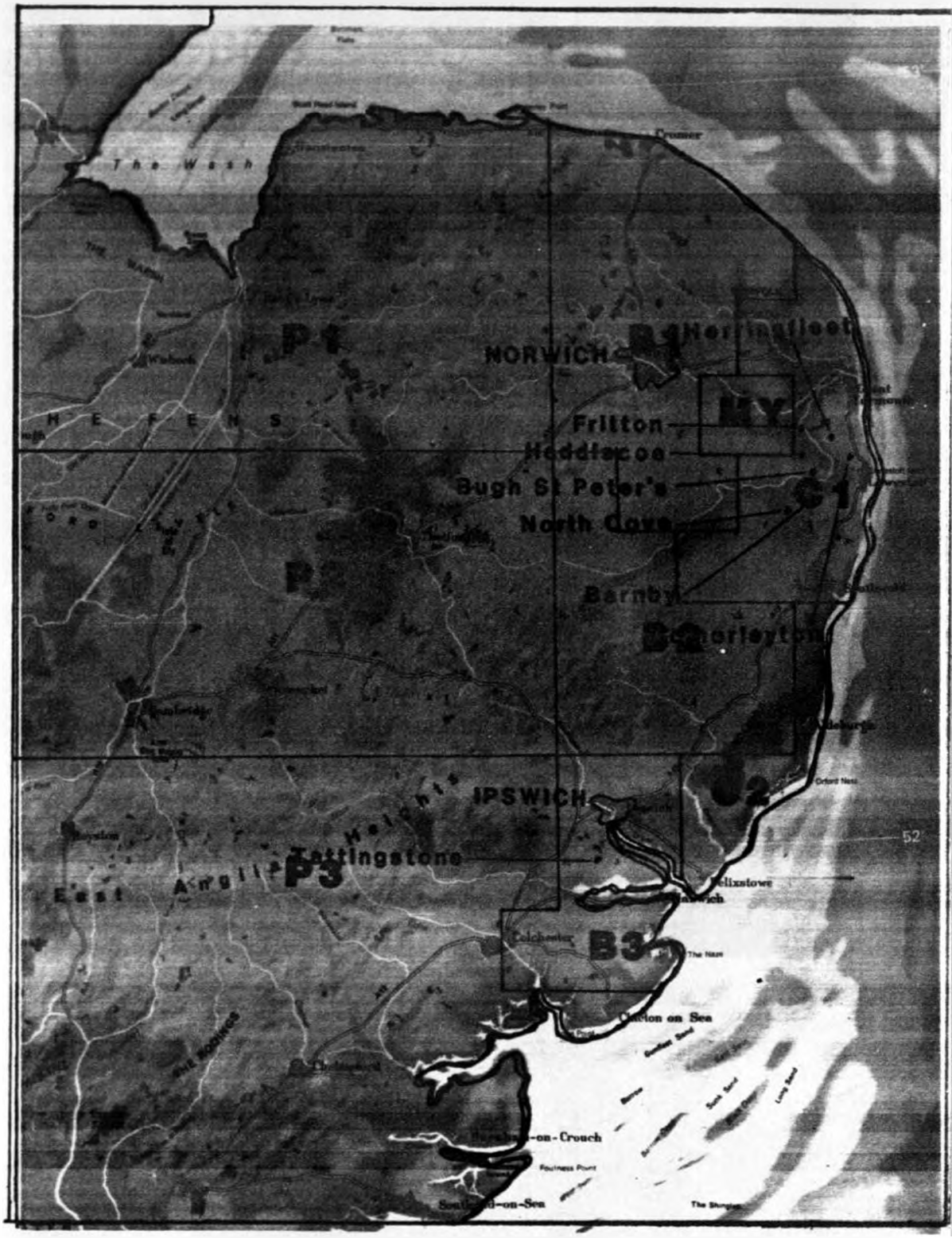

The Wash
P1
NORWICH
Herringfleet
MY
Fritton
Haddiscoe
Bugh St Peter's
North Cove
C1
Barnby
Lowestoft
P2
IPSWICH
C2
Tattingstone
P3
East Anglia
Felixstowe
Colchester
B3
Clacton on Sea
Burnham-on-Crouch
Foulness Point
Southend-on-Sea
52

FIGURE 9. Illustrates the typical terrain where coypu live. It is usually marshland with interconnecting ditches. The animals burrow into the banks of dykes and can cause conside rable damage by destroying the infrastructure of these water barriers.

FIGURE 10. Shows the type of trap used to catch coypu. It is usually placed in the known pathway of coypu and baited with a "tit bit" of either carrots or apples. The animal walks into the trap to eat the bait and the door closes behind entrapping the animal within.

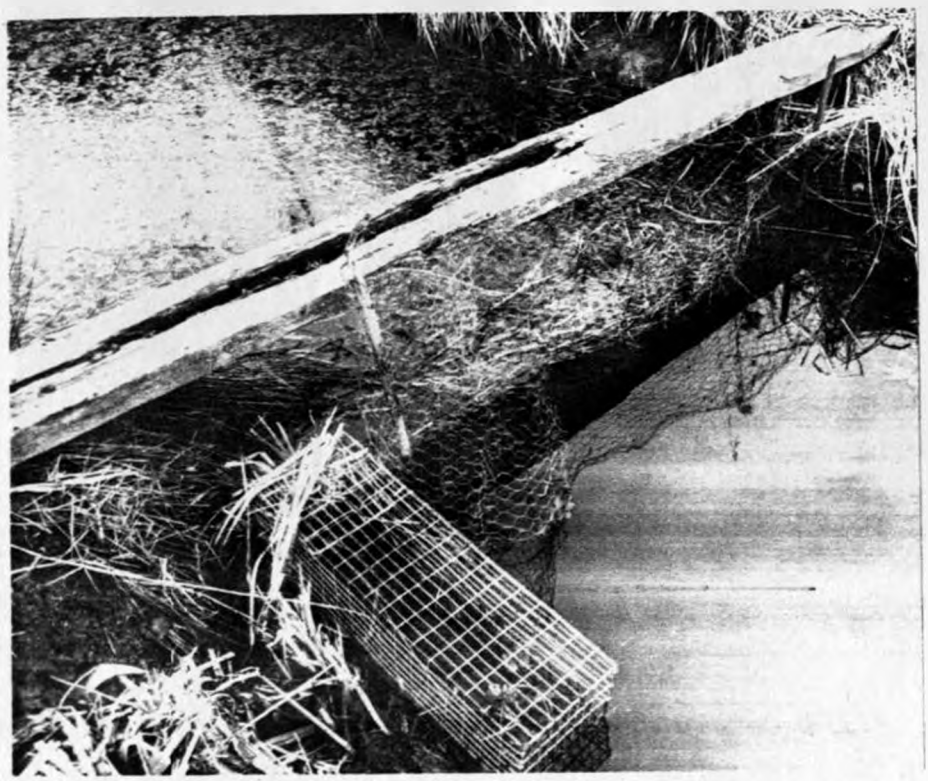
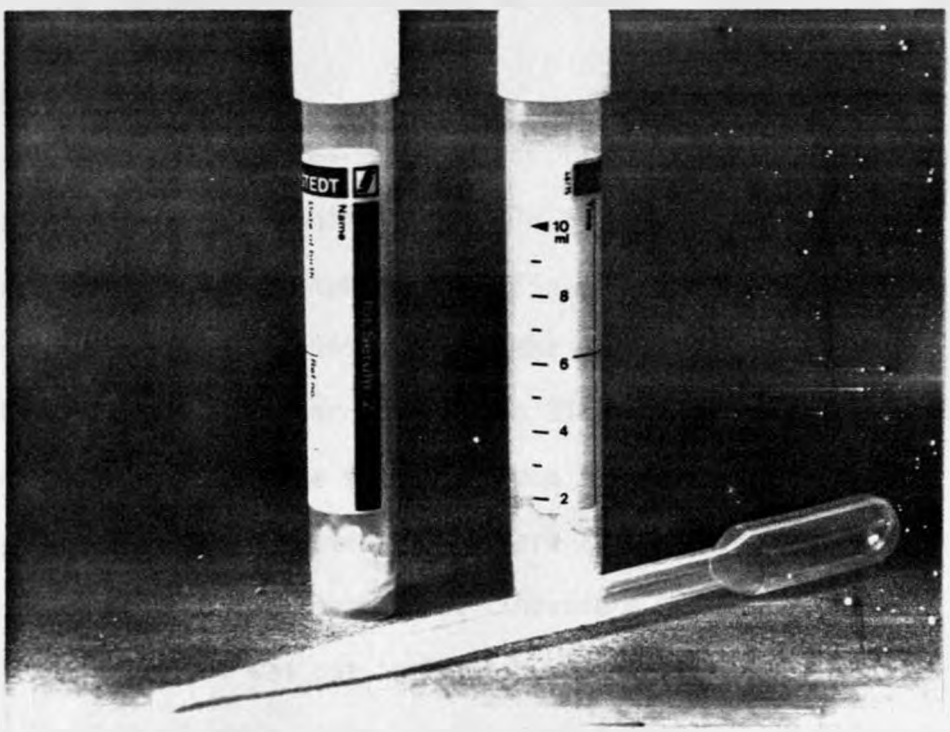


FIGURE 11. Shows the disposible tubes and a pasteur pipette used to collect blood and to process the resulting sera.



needle, about 1 ml of urine was withdrawn from the urinary bladder and about 0.5 ml put in a universal bottle with about 10 ml of Ellinghausen MacCullough media (EM) with 2% rabbit serum 0.1% agar and 0.02% 5-FU. By use of No. 21 disposable scapel blades and disposable plastic forceps, the kidneys were located, removed and sliced into two. About 0.5-1 gram of kidney tissue obtained from the cortico-medulla area of each kidney was put in a 5 ml syringe whose plunger had been removed. The plunger was put back and the tissue was gently squashed into EM with 2% rabbit serum, 0.1% agar and 0.02% 5-FU. Eyes were removed and together with the other halves of kidneys were put in plastic bottles with 10% neutral formalin *(Fig. 12)*.

(iii) Processing of specimens.

The kidneys and urine samples were each serially diluted by transferring about 3 drops from the universal bottle to about 3 ml media in bijou using disposable plastic pipettes *(see Fig. 13)*. Four serial dilutions were made on the spot into each of the following media; EM with 2% rabbit serum and 0.1% agar and EM with 2% rabbit serum, 0.1% agar and 0.02% 5-FU. The samples were stored at room temperature of 22-25^{o}C maintained by central heating system. They were 2 days later serially diluted from the fourth to the tenth tube. Therefore, each kidney and urine sample from an animal was put in 10 bijou bottles of media with 5-FU and 10 bijoux bottles of media without 5-FU, thus a total of 40 bijoux per animal. These were then brought to the laboratory and incubated at 30^{o}C. Before the above described media were used in the field, they were tested and found to support leptospiral growth.

Blood samples were allowed to settle at +4^{o}C and the clot formed in the tubes was released by wooden applicators and serum sucked using disposable plastic pipettes. Red blood cells were allowed to settle and again serum sucked. One drop of 4% sodium azide was added to each millilitre of serum to destroy possible becterial contaminants. The serum samples were brought to the laboratory and centrifuged at 2,500 rpm for 5 minutes. The

FIGURE 12. Depicts the formalised samples of eyes and kidneys collected during the survey.

The eyes were sent to the Coypu Research Laboratory, Norwich, to estimate the animal's age *(see Appendix III)*

The kidneys were processed for histological examination of leptospires *(see Figures 16a & b)*

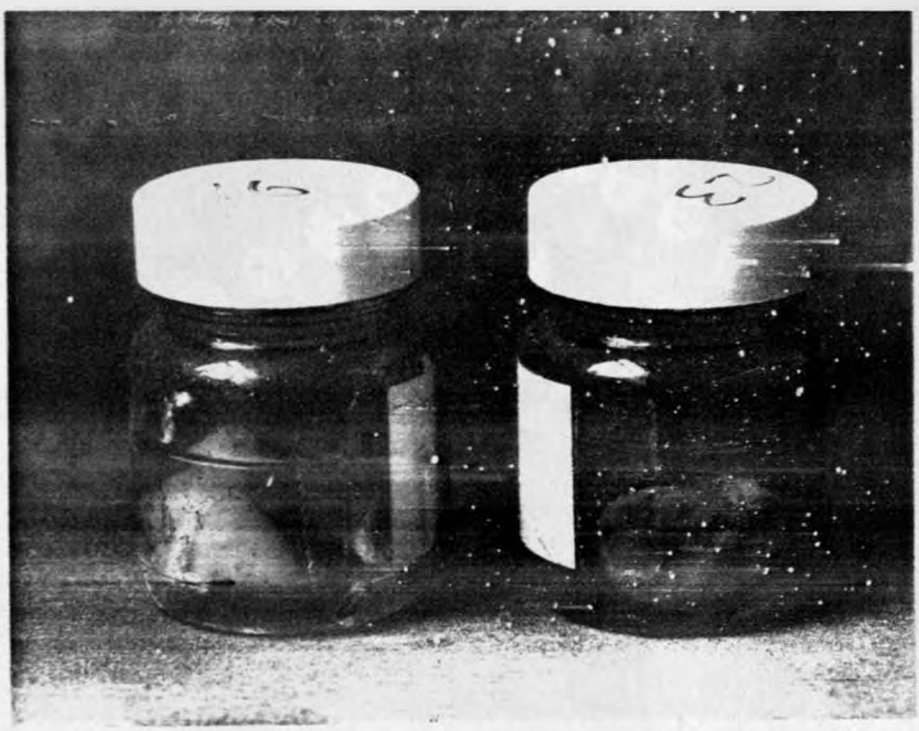
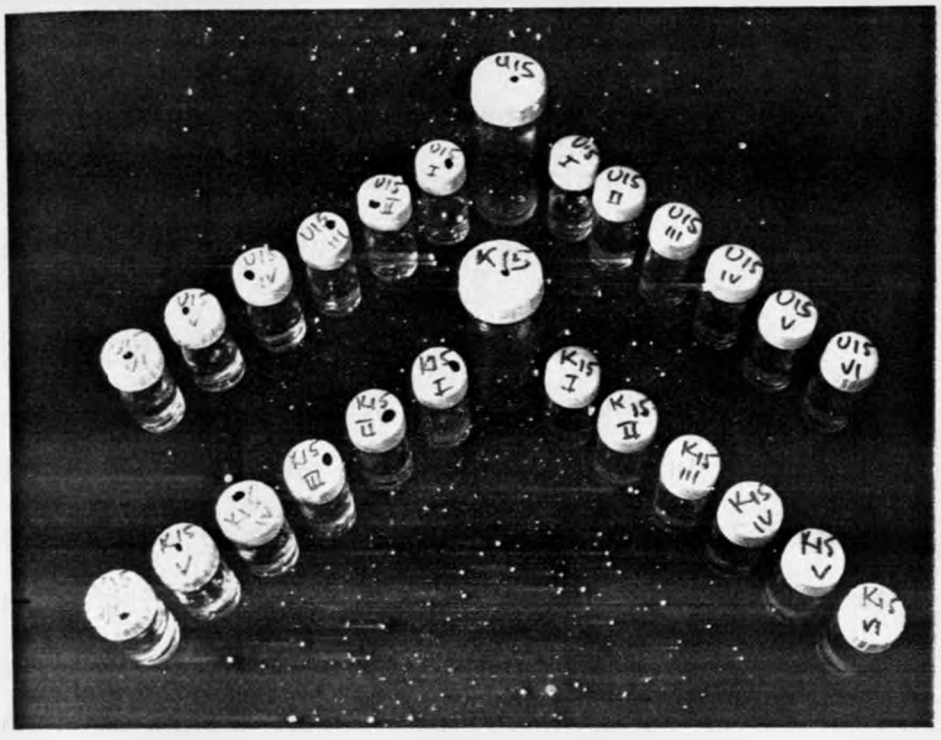


FIGURE 13. Illustrates both media used to grow leptospires from kidney and urine specimens. The photograph shows only some of the bijoux used. Those bijoux with black dots represent selective media which incorporated 5-FU and those without were non-selective media. Each specimen was serially diluted 10 times into both media. The total number of bijoux per animal was 40.



eyes were sent to the Coypu Research Laboratory in Norwich where the eye lenses were weighed and the age of the coypus computed *(Gosling, L.M., 1980)*. Eyes for coypu number six were not collected.

2. LABORATORY METHODS.

The following tests were performed on the sera obtained:

(i) Slide agglutination test (SAT), (ii), Cpmplement fixation test (CFT) and (iii), Microscopic agglutination test (MAT).

(i) Slide Agglutination Test (SAT)

The test done was that described by Mazzonelli, et.al. 1974 and modified by Coghlan. The antigen used in this test was *Patoc*. The coypu sera were tested neat and 1:10 dilution. The dilution 1:10 helps to overcome prozoning effect which may be misinterpreted as a weakly positive result. (See *Appendix IV* on the method of *Patoc* antigen preparation and the technical details of how the slide test was performed).

(ii) Complement Fixation Test (CFT)

A long time complement fixation test using $2HC_{50}$ units of complement, the optimal sensitizing concentration of haemolytic serum (HIB), 4% sheep red blood cells (SRBC) and a compound antigen was performed. (See *Appendix V* for the technical details of antigen preparation and the performance of the test). The end point was read as the highest dilution of serum that showed at least 50% fixation.

(iii) Microscopic Agglutination Test (MAT)

The sera were tested by the MAT at a dilution of 1:100 against 19 pools of formalized antigens. (See *Appendix VI* for the pools used and the technical details of performing MAT). These antigens represented a total of 19 serogroups. Sera with titres to pooled antigens were in a two-fold dilution system beginning at 1:80 to 1:20,480 titrated against each antigen within the positively reacting pool.

(iv) Examining the cultures.

Both urine and kidney samples were examined for leptospiral growth by dark field microscopy every two weeks for sixteen weeks.

(v) Histology.

The kidneys and liver sample were sectioned and stained with haematoxylin and eosin (HE) and Warthin-Faulkner silver impregnation stain method *(see Appendix VII* for technical details of staining procedures). A positive kidney specimen for control was prepared by inoculating 1 ml of *L.icterohaemorrhagiae* intraperitoneally into a Balb/c mouse at weekly intervals for two weeks and found to be positive by successfully culturing the leptospires.

(III) RESULTS AND DISCUSSION.

(i) Slide agglutination test results.

The SAT results show that coypu serum numbers, 5, 10, 15, 23, 27, 28 and 29 had very low antibody levels to leptospira detected only in neat samples and for practical purposes they are all reported negative *(see Table VII)*.

TABLE VII. Slide agglutination test results

Coypu serum number	Neat serum	1/10 serum
1	-	-
2	-	-
3	-	-
4	-	-
5	+	-
6	-	-
7	-	-
8	-	-
9	trace	-
10	+	-
11	-	-
12	-	-
13	trace	-
14	-	-
15	+	-
16	-	-
17	-	-
18	-	-
19	-	-
20	-	-
21	-	-
22	trace	-
23	+	-
24	-	-
25	-	-
26	trace	-
27	++	-

TABLE VII continued

Slide agglutination test results

Coypu serum number	Neat serum	1/10 serum
28	++	-
29	+	-
+ control	++++	++++

However, despite entering a negative verdict for leptospirosis basing on the nature of the results obtained from the above test, it is evident that at least some coypu had had some contact with leptospires.

Whereas *L.biflexa semaranga patoc* antigen has been known to cross-react well with pathogenic strains in the diagnosis of human cases of leptospirosis, it has on the other hand been known not to give satisfactory results with animal sera *(Addamino and Babudieri, 1968)*. The *patoc* antigen when used in screen test for animal sera may show varying concordance *(Eulogio et.al., 1978)*. It's usefulness in the wild animals as a screening antigen for leptospirosis may probably be of doubtful value.

Studies by Cacchione and his colleagues *(1971)* indicate that *L.biflexa semaranga rufino* may be a better antigen for slide tests in animals though this needs further research.

(ii) Complement fixation test results.

The CFT is a useful test in diagnosis of leptospirosis if only a rising titre can be demonstrated. However, in a screen test/survey like the one performed in this project, one cannot diagnose leptospirosis perse by use of CFT but can possibly only demonstrate the presence of antibodies to leptospires.

The CFT results presented in Table VIII show that all animals except

coypu number 14 had "antibodies to leptospira", unlike the SAT results.

TABLE VIII. Complement fixation test results

Coypu serum number	Titre expressed as reciprocal
1	160
2	80
3	80
4	160
5	160
6	80
7	160
8	80
9	80
10	80
11	80
12	20
13	80
14	0
15	320
16	40
17	20
18	40
19	40
20	40
21	80
22	320
23	40
24	80
25	40
26	40
27	80
28	40
29	80
Patoc control	1280

It must however be remembered that CFT results especially from animals are not reliable due to several factors, some of which are:- (i) the antibody antigen reactions (in both domestic and wild animals) do not fix guinea pig complement conventionally used in the indicator system, (ii) time elapsed since infection:- normally, CFT antibodies are detected about 10 days after onset of infection and they decline with time and, (iii) individual variation, e.g., failure to respond to infection by evoking CFT antibodies *(Sturdza., et.al., 1960, Nicolescu and Lelutiu., 1967)*. The failure to respond to infection may be is much more important in hosts which act as the reservoirs in that the organisms may be present without appreciable amounts of antibodies detected. This may be as a result of a stable host-parasite relationship (iv) CFT sometimes may cross react with unrelated antigens to leptospira.

However, if a titre of 1/100 is assumed as the base minimum after which an animal is considered positive *(Michna, S.W., 1967)*., then six animals, i.e., coypu numbers 1, 4, 5, 7, 15 and 22 are positive. However, the CFT results compared with the SAT results obtained in this project seem to bear little relationship to each other.

(iii) Microscopic agglutination test (MAT) results.

The 1/100 dilution screen as the minimum at which serum was considered positive revealed that coypu serum numbers 2, 3, 5, 7, 9, 15 and 22 were positive *(see Table IX)*. Results obtained after further titration against the antigens they reacted with are shown in Table X. From these, it is quite evident that all the MAT positive samples, except 15, had antibodies to *L.icterohaemorrhagiae*. Coypu humber 5 and 15 had antibodies to more than one serogroup *(see Table X)*

(a) TABLE IX. MAT RESULTS of 1/100 serum dilution.

Serum No.	Antigen pools 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	+	-	-	-	-	-	*tr*	-	-	-	-	-	-	-	-	-	-	-	-
3	+	-	-	-	-	-	*tr*	±	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*tr*
5	+	-	-	*tr*	-	-	+	+	-	-	-	-	-	-	-	-	-	-	*tr*
6	-	-	-	-	-	-	-	-	-	-	*tr*	-	-	-	-	-	-	-	-
7	+	-	-	-	-	*tr*	-	-	-	-	*tr*	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	*tr*	-	-	-	-	-
9	+	-	-	-	-	-	*tr*	*tr*	-	-	-	-	-	-	-	-	-	-	*tr*
10	-	-	-	-	-	-	-	±	-	-	-	-	-	-	-	-	-	-	*tr*
11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*tr*
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*tr*
13	-	-	-	-	-	-	*tr*	-	-	*tr*	-	-	-	-	-	-	-	-	*tr*
14	-	-	-	-	*tr*	-	-	*tr*	-	-	-	-	-	-	-	-	-	-	*tr*
15	*tr*	-	-	*tr*	*tr*	+	*tr*	-	-	-	+	-	(±)	(±)	(+)	-	-	*tr*	-
16	-	-	-	-	-	-	*tr*	-	-	-	*tr*	-	-	-	-	-	-	-	(±)
17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(±)
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*tr*	-	-	-	*tr*
19	-	-	-	-	-	-	-	-	-	-	*tr*	-	-	-	-	-	-	-	(±)
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	*tr*	-	-	-	-	-	-	-	-	-	-	-	-
22	+	-	-	-	-	-	*tr*	*tr*	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-	(±)	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-	-	*tr*	-	*tr*	*tr*	-	-	*tr*	*tr*
26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*tr*
27	-	-	-	-	-	-	-	-	-	-	*tr*	-	-	-	-	-	-	-	*tr*
28	-	-	-	-	-	-	*tr*	-	-	-	-	-	*tr*	-	-	-	-	-	*tr*
29	*tr*	-	-	-	-	-	*tr*	-	-	-	-	-	-	-	-	-	-	-	(+)

See Figs, 5a, b, c and Appendix VI for interpretation of the readings.

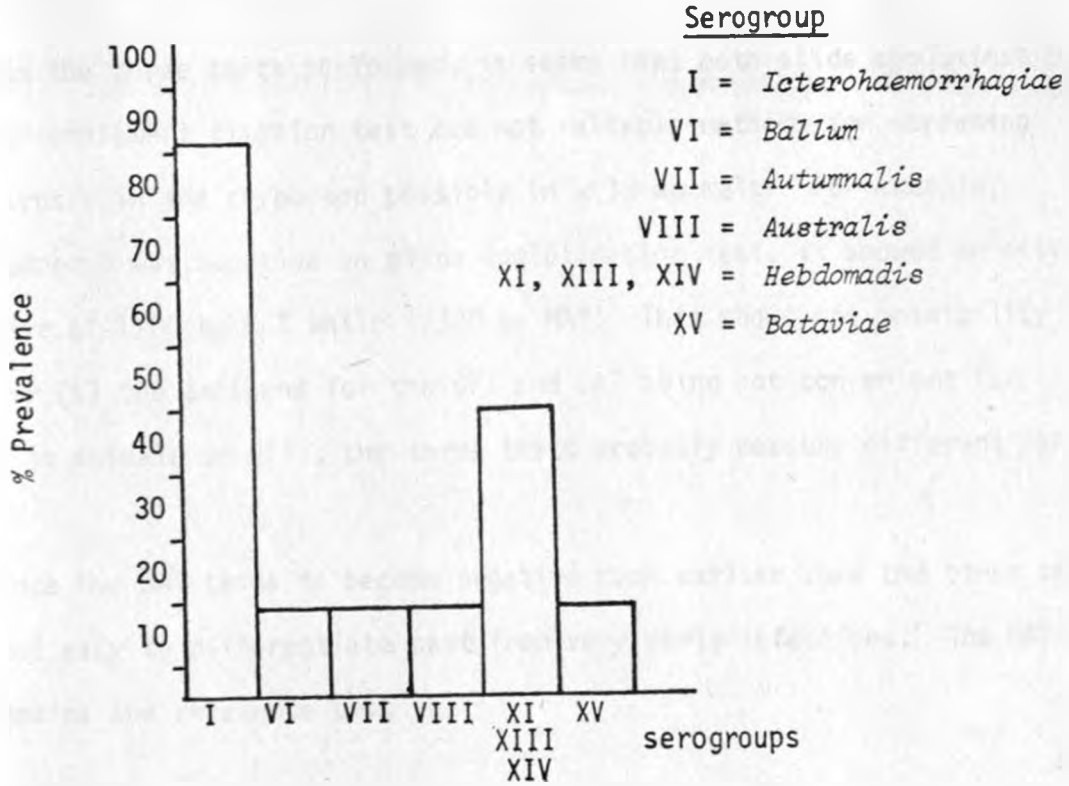
+ = complete agglutination. (+) = 60 - 90% agglutination. ± = 50% agglutination.
(±) = 20 - 40% agglutination. *tr* = trace. - = NO agglutination.

(b) TABLE X. Results of positive sera (above) after dilution and titration against the appropriate antigens. Titres given as reciprocals.

Serum number	Antigen pools I	VI	VII	VIII	XI	XIII	XIV	XV
2	160							
3	320							
5	640		320	80				
7	320							
9	160							
15		160			1280	80	80	160
22	160							

The MAT is the reference test for the diagnosis of leptospirosis *(Turner, L.H., 1968a)*. From the results recorded above and from results in *Fig. 14* and *Table XI* below, it seems as though *Icterohaemorrhagiae* and *Hebdomadis* serogroups have a high serological prevalence in the coypu.

Figure 14: Serological prevalence in the coypu (in only 7 positive animals by MAT).



There seems to be no relationship between sex, age and serological incidences. Seven out of 29 coypus (24%) had serological evidence of leptospirosis by the MAT test. The overall breakdown of the serological incidences is shown in the Table XI, below:

TABLE XI. Percentage prevalence by serogroup.

Serogroup	Number positive against serogroups: (a) +ve cases at 1:80	(b) Total No. of coypu	Percentage per +ve case	Percentage per population
Icterohaemorrhagiae	6/7	6/29	86%	21%
Ballum	1/7	1/29	14%	4%
Autumnalis	1/7	1/29	14%	4%
Australis	1/7	1/29	14%	4%
Hebdomadis *	3/7	3/29	43%	10.3%
Bataviae	1/7	1/29	14%	4%

* = including *Sejroe, Wolfii* and *Mini* only.

From the three tests performed, it seems that both slide agglutination test and complement fixation test are not reliable methods for screening leptospirosis in the coypu and possibly in wild animals. For example, serum number 3 was negative on slide agglutination test, it showed an antibody titre of 1/80 by CFT while 1/320 by MAT! This shows the possibility of either (i) the antigens for the CFT and SAT being not convenient for surveys in animals or (ii), the three tests probably measure different parameters.

Since the SAT tends to become negative much earlier than the other tests it is not easy to differentiate past from very early infections. The MAT thus remains the reference test.

(iv) Cultural examination results.

Leptospires were observed in urine culture of one coypu (coypu number 13)

after 49 days incubation. Growth occurred in only one bijou with 5-FU that represented the first dilution from the main universal bottle. The isolate was immediately passaged into Ellinghausen McCullough media with 2% rabbit serum and into DIFCO media. The organism grew better in the former. It is of interest that the isolate which was recovered was isolated from the oldest of all the coypus caught; (1333 days old - see *Appendix III*).

To determine if the isolate was pathogenic, it was passaged in 8-azaguanine; first in 225µg/ml, then 7 days later in 400µg/ml and examined after 10 days. *Patoc*, *icterohaemorrhagiae* and *hardjo* strains were used as controls. *Icterohaemorrhagiae*, *hardjo* and the isolate did not grow in 8-azaguanine on secondary passage while *patoc* did, which indicated that the isolate was pathogenic. The leptospiral isolate was subject to the Kmety identification scheme and was found to react with *hebdomadis* and *Sejroe* serogroup equally. Further identification studies by cross-absorption will be done on the isolate to determine the exact identity. However, the performance of the identification procedures requires several months and it is not possible to include the exact identification of the organism in the span of this thesis and therefore, the identification of this isolate will only be reported to serogroup level.

(v) Histological results.

Histological examination of haematoxylin and eosin (HE) stained kidney sections revealed no pathological evidence of leptospirosis in any of the coypu examined *(see Fig. 15)*. Results obtained by the Warthin-Faulkner silver impregnation technique revealed the presence of leptospiral organisms in the kidney of coypu number 30 which was also culture positive *(see Fig. 16 a & b and Addendum on page 67)*.

It must be noted that although the silver staining techniques are useful in demonstrating the presence of leptospiral organisms in tissues, I have found that these methods are probably better for tissue smear preparations than thin sections, because the latter may be thick and stain densely thus concealing the leptospires. This is indirectly substantiated by the work of Burdin, M.L., *(1963)*. In retrospect, impression smears should have been prepared during the field survey in addition to formalin fixed tissues for histology. Despite this, it has been possible to demonstrate with difficulty the presence of the organisms in the kidney sections of the coypu *(see Fig. 16 a & b)*.

The demonstration of leptospiral organisms in renal tissue is of diagnostic value. In man and domestic animals, it may reflect a subclinical infection but be unrelated to the pathogenisis of the disease. In wild animals, the presence of leptospires in the kidney, even in the absence of any pathological features, indicates that the animal may be a potential reservoir of infection.

In general, the demonstration of leptospires in the kidney and other organs, for example the liver or aborted foetuses with serological evidence of leptospirosis, may substantiate a diagnosis of the disease. The concurrent application of histological and serological methods reinforces clinical observation.

(vi) Discussion.

From the results obtained it is apparent that the coypu harbours

FIGURE 15. Shows a Haematoxylin and Eosin (HE) stained section of a coypu kidney from which leptospires were isolated. The pathology appears to be normal. However, if Warthin-Faulkner silver impregnation technique is used, leptospires may be seen in the tubules *(see Figures 16a & 16b)*.
Magnification X 400.

FIGURE 16a. Shows a Warthen-Faulkner stained section of the same kidney as seen in Figure 15. Aggregates of leptospires are seen in the tubules (†).
Magnification X 1000.

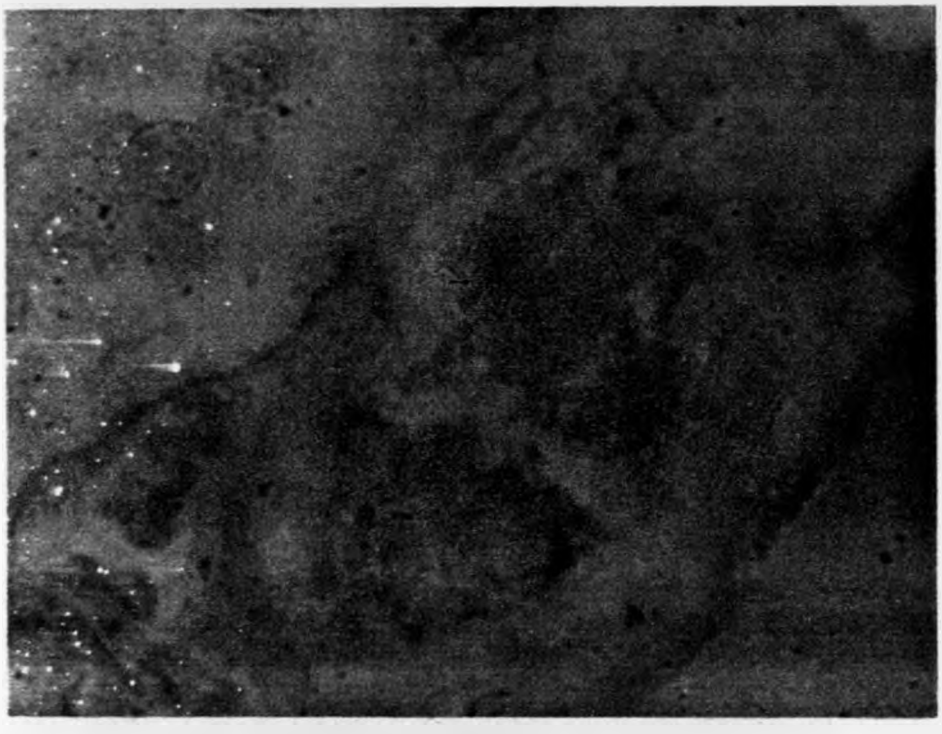
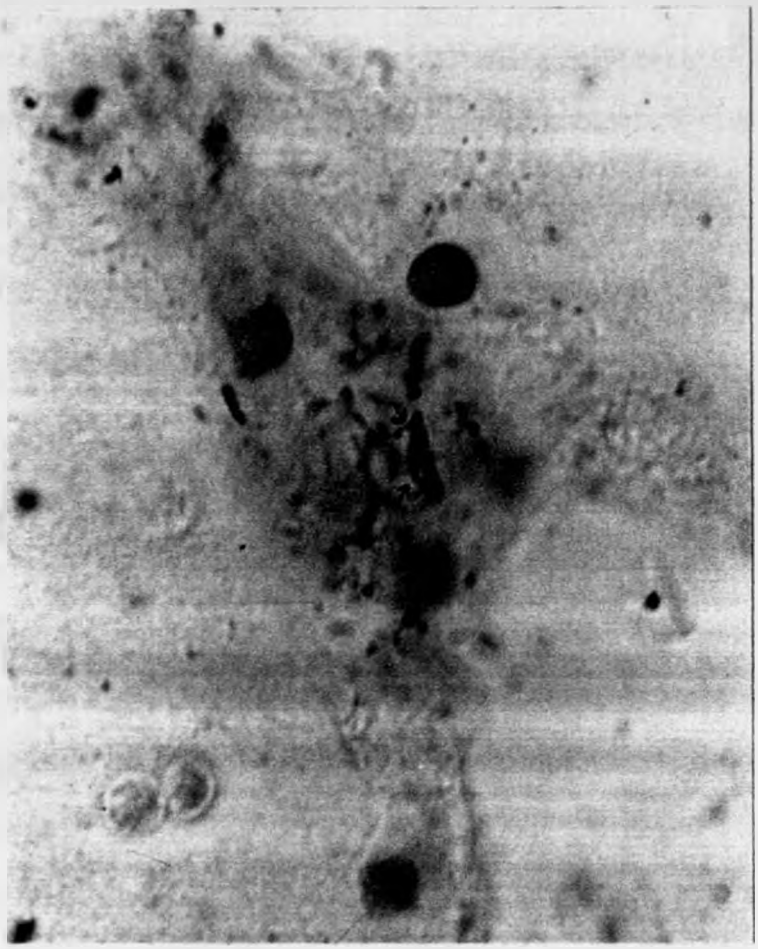


FIGURE 16b. Shows a more detailed view of the tubule contents. Silver stained leptospires can be seen (↑). Magnification X 1000.



leptospira. This has been established from studies in Argentina, United States of America (U.S.A.) and in the Union of the Soviet Socialist Republics (U.S.S.R.). This project appears to be the first report of the isolation of leptospira from the coypu *(Myocastor coypus* Molina) in Great Britain. Several workers have demonstrated the presence of leptospiral anitbodies in the coypu in this country. This project also appears to be the first report of isolation of *hebdomadis* or *sejroe* serogroups in the coypu. Since the exact serogroup of the isolate has not been declared *(hebdomadis* and *sejroe* are very closely related), it appears that if the isolate was *sejroe*, then this project has revealed a reservoir for *Hebdomadis* infections in domestic animals in the East Anglia region where sporadic outbreaks of the disease in cattle has been thought to be as a result of contaminated rivers and canals by infected cattle on distant farms up-stream. It appears that the coypu may be a reservoir of *Hebdomadis* infections and could contribute to the cattle associated leptospiral outbreaks.

Attempts to isolate leptospires from wild animals are usually part of surveillance programmes and the majority of the trapped animals are normally clinically healthy. Therefore, only serovars capable of colonizing the kidney for long periods of time are usually isolated from such animals. In this project, it has been possible to isolate for the first time a leptospiral strain from the coypu that was healthy and whose histological sections of kidneys show no evidence of pathology. It is, therefore, possible that the coypu is a reservoir for leptospirosis in Great Britain.

Generally when the cultural and positive serological rates for a species are equal, it indicates the presence of persistent infections and hence the animal is thought to be a good carrier. However, when more animals have antibodies than isolation or histological rates, the indication may be that the species has fleeting infections and leptospires are not excreted for long and therefore, the species is probably not a good carrier. However, several investigators have isolated leptospiral organisms from animals which

had no evidence of leptospiral antibodies *(Babudieri, B., 1958a, Broom, J.C., and Coghlan, J., 1958, Menges, R.W. et.al., 1960, Minette, H.P., 1961, Meridian, G.B., 1966, Mackintosh, et.al., 1980, Thierman, 1983 etc.)* Similar studies were demonstrated by Roth, E.E., et.al. *(1962)* in the coypu.

The phenomenon of culturally positive and serologically negative animal reservoirs has been partially explained by Babudieri, *(1958)*. in a theory of "ectoparasitism". The theory postulates that an initial stage of leptospiraemia is followed by an immunized state. The organisms accumulate in the secondary convulated tubules of the kidney and at this point the leptospires stop being internal parasites. The organisms stop stimulating the antibody forming mechanism of the parasitized animal and conversely, the previously formed antibodies in the blood stream cannot affect the organisms. The explanation for this is not yet fully understood. The blood antibody level gradually diminishes until it reaches a point where it is no longer detectable by present serological methods. Leptospiruria may persist, sometimes for the life of the animal, and these "carrier animals" thus become one of the potentially dangerous sources of infection.

Faine *(1957, 1962a, 1962b)* in his studies of the carrier state found that very young rodents quite often died from the infection, while older rodents were apparently more resistant and became carriers, possibly as a result of the rate of antibody production. His further experimental work on mice indicated that, the carrier rate condition resulted from the ability of the virulent leptospirae to (a) grow in the host and produce lesions in the primary, acute generalized infection, and (b) grow in the renal tubules in the presence of antibodies that could pass from the blood stream through the renal tubules into the urine.

In situations where there is a reservoir of known wild animal species which has been proven negative both serologically and bacteriologically, it is probable that in that particular location the animals are genetically resistant to leptospira as a result of their geographical isolation. This

has been demonstrated by Chernukha, et.al. *(1975)* in the U.S.S.R field mice's susceptibility to serovar *mozdok* and has been suggested by Hathaway and his colleagues *(1982c)* as the possible case in Great Britain's house mice inability to harbour leptospira.

In this project it may be concluded that the coypu is a reservoir of leptospires in Great Britain and therefore a potential source of infection for both man and domestic animals. However, compared to other animal reservoirs population and distribution in Great Britain, the coypu may be of little significance. However, it"s presence in rivers of East Anglia may present a hazard to those pursuing water sports and because of this the coypu may be an important reservoir for both humans and animals in this area.

SECTION B

CONTENTS

(I) INTRODUCTION TO SECTION B

Following the results obtained by the *patoc* slide agglutination test (SAT), complement fixation test (CFT) and the microscopic agglutination test (MAT) in the screening for leptospirosis in the coypu *(Myocastor coypus* Molina) described in Section A of this project, it became apparent that both the *patoc* slide agglutination test (SAT) and the complement fixation test (CFT) were not suitable for screening animals for leptospirosis since the tests did not correlate. It was decided therefore, that a comparison of *patoc* SAT and the commercial DIFCO SAT be done on coypu serum samples and extend the comparison to human and bovine serum samples. A microscopic agglutination test (MAT) was carried out on all the samples to determine positive from the negative samples as MAT is the reference test *(Turner, L.H., 1968a)*. The two tests; *Patoc* and DIFCO were thus compared with MAT as the reference.

(II) MATERIALS AND METHODS

40 human serum samples (supplied by the Leptospira Reference Laboratory, of which 11 were known to be from positive cases), 26 coypu serum samples (obtained from the project in Section A) and 32 bovine serum samples (from Iraq) were tested. The DIFCO slide agglutination test antigens were purchased from the DIFCO Laboratories, Surrey, England, while *patoc* slide antigen was prepared as described in Appendix IV. The *patoc* test and DIFCO tests were carried out as specified in Appendix IV and VIII respectively. The microscopic agglutination test was carried out as described in Appendix VI.

(III) RESULTS AND DISCUSSION

The results of the tests are recorded in Appendix IX and are analysed in Tables XIII, XIV and XV below. Table XIII shows the numbers and the percentage of positive cases recorded by each test.

TABLE XIII. Percent positive by each test.

Species	Number examined	Positive by the following tests:			Percentage positive by following tests:		
		MAT	Patoc	DIFCO	MAT	Patoc	DIFCO
Coypu	29	7	0	2	24%	0%	6.8%
Man	40	11	8	11	27.5%	20%	27.5%
Cattle	32	17	20	14	53%	62.5%	44%

Table XIV below shows the breakdown of the positive cases by each test.

TABLE XIV. Breakdown of the positive samples by each test.

Species	+Ve by MAT	+Ve by Patoc	MAT and +Ve only by DIFCO	Patoc	DIFCO	+Ve by MAT alone	+Ve by both Patoc & DIFCO
Coypu	7	0	2	0	0	5	0
Man	11	8	10	0	1	0	0
Cattle	17	11	8	7	3	5	3

From the above table, it is apparent that some specimens were positive either by; MAT, *Patoc* slide test or by the DIFCO slide test. Table XV shows the percentage concordence of the *Patoc* and DIFCO slide tests with MAT. MAT is the reference test.

TABLE XV. Percent concordence with measures of "false positives" and "false negatives."

Species	+Ve by MAT	+Ve by Patoc	MAT and Patoc	Percent concordence with MAT		Percent "false +Ve"		Percent "false -Ve"	
				Patoc	DIFCO	Patoc	DIFCO	Patoc	DIFCO
Coypu	7	0	2	0%	29%	0%	0%	57%	42%
Rat	11	8	10	73%	91%	0%	9%	0%	0%
Cattle	17	11	8	65%	47%	41%	18%	0%	6%

$$\% \text{ Concordence} = \frac{\text{No. positive by MAT and Patoc/DIFCO}}{\text{No. positive by MAT}} \times 100$$

Coypu

From the results obtained, it appears that both *Patoc* and DIFCO slide tests are not as reliable as the MAT in screening the coypus for leptospirosis. Based on the criteria used *(see Appendix IX)* to determine a positive and doubtful results from negative ones, DIFCO slide test detected only two animals as positive, *Patoc*, one and MAT, seven. However, all three tests did not detect the presence of antibodies in coypu number 13 from which an isolate was made (see previous Section A). The failure to detect antibodies in carrier animals has been observed by many investigators including, Minette, H.P. *(1964)*, Meridian, G.B. *(1966)* etc. In a survey conducted by Minette, H.P. *(1964)* in Hawainian rodents and mongooses, the SAT (DIFCO's first four pools) failed to identify 56.3% of the culturally positive rats. In his study, he showed that leptospires could be correctly detected both culturally and serologically in mice, but that in mongooses there were twice as many seropositive animals than culture positive. This probably demonstrates the variability of serological tests with different animal species. Detailed information of this kind on the coypu is lacking and it is therefore not scientifically unusual to rely

on serological tests.

Considering that animals which actually harbour the living organisms constitute the greater hazard in transmission of the disease to both animals and man, it is clear that the epidemiological surveys using culture techniques are more reliable than those utilising only serological examination for the detection of the evidence of leptospirosis in wild animals.

Man.

There is a good correlation between the DIFCO agglutination test and MAT in diagnosing positive patients (91% concordence; see Table XV). The *Patoc* test showed only a 73% concordence. However, it would be inaccurate to assume that the *Patoc* test is inferior to that of DIFCO's because the criteria used to define a positive from a negative result, i.e. the minimum antibody level measured by the MAT at 1:40 does not allow for the diagnosis of the very early rise in antibody level which the *Patoc* test can measure in the early phase of the disease. This may probably account for several samples being grouped as "doubtful" with the *Patoc* test. The absence of "false positives" by the *Patoc* test may, however, be misleading because the samples tested are small. In addition, the samples used to assess the different slide tests were from patients with a known record of leptospirosis and, therefore, the absence of false positives may be a true measure. Although the DIFCO test showed only 9% "false positive" *(see Table XV)*, it did have a good concordence value and may be used as a screen test for leptospirosis in man. It must be pointed out that in some cases there was no correlation between the reactive pools and the known infecting serogroup *(see Appendix IX)*.

Cattle.

Whereas *Patoc* slide test showed 65% concordence with MAT and DIFCO only 47%, the *Patoc* test also showed many false "positive reactions" and

the DIFCO test less. With such results, it is not easy to determine which of the two is the better test. Studies by Nicolescu, et.al., *(1982a and 1982b)* on larger samples have indicated that the *Patoc* test may be used in epidemiological enquires in animals and man as it showed reasonably high enough concordence with the MAT; the reference test. In this project a small number was available for the test but it is evident that there was a better concordence between the *Patoc* and MAT than DIFCO and MAT. The presence of many "doubtful" reactions with the *Patoc* test may have the same explanation as outlined above for man.

Discussion:

The extreme variability in clinical symptoms and lack of pathognomonic lesions in most cases of human and animal leptospirosis make isolation of the microorganism and /or serological tests an absolute requirement for the definite diagnosis *(Torten, M., 1979)*. The serological tests available for the diagnosis of leptospirosis include the following; slide agglutination test (SAT), microscopical agglutination test (MAT), complement fixation test (CFT), enzyme linked immunosorbent assay (ELISA), sensitized erythrolysis (SEL), immunofluorescent techniques and haemagglutination test (HA)., etc.

The MAT is still the reference test used in serological diagnosis and identification of leptospirosis *(Turner, L.H., 1968a)*. However, it is a prerequisite that the laboratory maintain a large stock of cultures. It is also a time consuming and tedious procedure to perform especially when a wide ranging number of antigens are used with each serum sample to be tested. On the other hand, the slide agglutination test (SAT) described first by Galton and her colleagues *(Galton, M.M. et.al., 1958)*, now commercialized as DIFCO slide test, is considered a reliable screening test for detection of acute and recent infection by leptospires in many laboratories. However, like MAT, it requires the use of more than one antigen.

Because of the little importance attached to knowing the infecting

serovar in the management and treatment of a case by clinicians and veterinarians, a straightforward test for the "diagnosis of leptospirosis" is adequate. Therefore, the Mazzonelli - Mailloux slide test *(Mazzonelli, et.al., 1974)* modified by Coghlan is preferred by some laboratories. The test uses a formalin killed and concentrated *Patoc 1* as the sole antigen. *Patoc 1* cross reacts with all known pathogenic leptospires and has shown a very high concordence with MAT in the diagnosis of human leptospirosis; up to 90-98% concordence. *(Elian, M. and Nicoara, I., 1965, Correa, M.O.A., et.al., 1970).* In this project, it was possible to demonstrate a 73% concordence. The sample number in this project was however, small and probably a larger sample number would give results in the range of 90-98%.

In trying to decide upon which test can be recommended for a diagnostic screen, it is inevitable that the economic aspects of each test be considered. The costs of running *Patoc* and DIFCO slide tests are therefore calculated below, which were corrected at the time of the project.

Patoc test.

10 mg bovine albumin @ £314 per 500 mg	£8. 00
10 ml rabbit serum @ £28 per 2 litres	£0. 14
Others, less than	£2. 00
Approximate cost	£10. 00

Total amount of antigen from 1 litre culture ..... 20 ml

Amount used per test: 40µl (20µl for neat and 20µl for 1:10 dilution)

Total number of tests possible to run from 20 ml .... 500 tests

Cost per test £0. 02

DIFCO test

6 pools of antigens @ £15 per pool	£90. 00
Total amount of antigen	30 ml
Total possible number of tests from all the pools ...	100 tests
Cost per test	£0. 90

NB: Not all the pools of antigen are in stock at any one time and difficulties in acquiring these pools is encountered. It took 70 days to get all the pools.

In conclusion, I have shown that serological tests are not an accurate measure of the carrier rates in wild animals as they may not give a true epidemiological picture. Cultural methods, though tedious and delicate for inexperienced personnel, give more accurate results. As regards the diagnosis of the disease in man, both DIFCO and *Patoc* tests are equally useful. However, if the economic costs and the conveniences of the tests are taken into account, then the *Patoc* slide test has advantages over the DIFCO slide test.

SECTION C

GENERAL DISCUSSION

GENERAL DISCUSSION.

I have demonstrated in this project that the coypu (*Myocastor coypus* Molina) harbours leptospires, and have isolated the organism from both live and dead coypu. This is the first time in Great Britain that leptospires have been recovered from this species of animal. Based on the numbers examined, the serological incidence for leptospirosis in the coypu is 24%, while the carrier rate is about 4%. There is a high serological incidence to *Icterohaemorrhagiae* and *Hebdomadis* serogroups; 86% and 43% respectively. In view of the recently noted increase in the cattle associated leptospirosis (CAL) in man in East Anglia (12%) with no corresponding changes in the mice and rat population in the area, it is possible that the coypu plays a role in the epidemiology of the cattle related disease in East Anglia. As to what the exact extent the animal plays in the role of reservoir for human and domestic animal infections generally is still unknown.

In this project, I have also demonstrated that cultural techniques are a better index for detection of carrier animals in cases where detectable levels of antibodies are not seen.

A comparison of the *Patoc* and DIFCO slide tests for screening leptospirosis in man shows that the two tests are useful and may be used as screening tools, however, the price of the DIFCO product may be prohibitive.

Leptospirosis is classified as a direct arthropozoonosis. The epidemiology of the disease is a constantly changing dynamic process whose pattern is not entirely understood. A shift in the host-parasite relationship renders varying relative importance to new and old hosts, hence a serovar that may not be clinically associated with certain groups of people or animals may over the seasons and years become the most commonly found infecting strain.

At any given point in time, scanning the world for the pattern of human leptospirosis will undoubtedly reveal the presence of the different

epidemiological patterns *(Torten, M., 1979)*. These changes are usually accompanied and/or preceded by changes in epidemiological conditions. Under such conditions, the pattern of human and animal diseases change. It has already been observed that there is a tendency for a global shift to *L.Hebdomadis hardjo* (now reclassified as *L.Wolffii hardjo)* infection in dairy and cattle associated populations *(Shenberg, E., et.al., 1982, Van der Hoeden, 1964, etc.)* New Zealand, Roumania, Israel, U.S.A. and now Britain and possibly Holland are currently undergoing epidemiological situations in which most human infections are due to contact with domestic animals *(Dis, D.R., et.al., 1973, Nicolescu, M. and Moldoveanu, G., 1974, Shenberg, E., et.al., 1977 and Kaufman, A.F., 1976)*. At the same time, Japan and most developing countries including Jamaica, Barbados, Nicaragua and Kenya are relating most of their human infections to contact with wild hosts, especially of the order Rodentia *(Everard, C.O.R., et.al., 1976, Sufres, B., 1976 and Kranendonk, et.al., 1969)*. In fact the epidemiology of leptospirosis in the developed and developing countries may be dissimilar.

Each country has it's own endemic zones that fit the criteria required for leptospiral survival outside the host. The most important source for human infection in any given locality in any country may be quite different from that of the domestic animals living in the same locality. The mere presence of large numbers of carrier animals in any particular area does not necessarily mean that they are the most important hosts for human infections there. Some other hosts in small numbers that are present but carrying human virulent serovars could be the most important *(Torten, M., 1979)*. Despite this knowledge about the epidemiology of leptospirosis, information is lacking on the relative importance of host-to-host interaction in causing shifts in leptospiral virulence in man and domestic animals.

In general, the desert areas of the world should not be considered potential endemic zones. However, with the increase in man made lakes in

such areas and development of irrigation schemes which are most prevalent now in the third world, there is a tendency for man to be in close contact with domestic and wild animals that concentrate in such areas. Thus such irrigation schemes do not only tend to increase food production (the primary aim) in any such countries but also tend to create focal points for many water borne or associated infections including schistosomiasis, malaria, cholera and also leptospirosis.

The number of synonyms given to leptospirosis throughout the years and in many parts of the world *(see Appendix X)* serves to exemplify the confusion that the clinical symptoms present to doctors, veterinarians and researchers alike. Since leptospirosis can be so variable in its symptomatology, it should be considered as a differential diagnosis in almost every case of pyrexia of unknown origin (PUO) whether or not it is accompanied by severe or mild combinations of symptoms affecting any organ system.

In the laboratory, the diagnostic techniques available for leptospirosis include microscopy on the specimens, cultural or serological methods. None of these are ideal as a diagnostic tool for leptospirosis in all situations of the disease. This is not as a result of the inherent inadequacies of the procedures but due to the features of leptospiral infections. The disease runs a course of leptospiraemia which precedes the febrile stage by a few days and is followed by leptospiruria which is accompanied with antibody production that reaches a peak titre in the second to third week; after which their longevity in the body depends on individual species *(Roth, E.E., et.al., 1961b)*.

The differences in the immunoglobulin components of antibody provoked in animals and man may relate significantly to the sensitivity of serological procedures, e.g., CFT, haemolytic tests, etc., *(Bull.Wld.Hlth.Org., 1972)*. Due to the variables inherent in serological tests and the variation in antibody response of hosts in leptospiral infections, serological test results must be considered case by case and evaluated in relation to all

other data, particularly clinical observations.

In domestic food animals, leptospirosis is usually a herd problem and, therefore, serological results of a representative number of animals when evaluated in the light of clinical and pathological findings are very valuable in arriving at a diagnosis. However, differential diagnosis should be considered. Negative results for the differential tests strengthens positive serological findings for leptospirosis. Therefore, when serological tests in both man and animals are interpreted in their proper perspective, they constitute a valuable laboratory aid in the diagnosis of leptospirosis. The demonstration of a significant rise in antibody titre affords excellent evidence of recent infection.

Leptospirosis is best prevented by active immunization *(Little and Hathaway, 1982)* and although leptospiral vaccines are amongst the most effective vaccines for preventing clinical signs of disease, they can lead to creation of assymtomatic renal carriers which may infect other animals, including man *(Feigin et.al.,1973)*. Furthermore, since leptospiral vaccines are usually prepared from cultures grown in media containing serum protein, they occasionally cause hypersensitivity reactions. Following the development of protein free synthetic leptospiral growth medium, *(Shenberg, E., 1967, etc.)*, it is possible to prepare vaccines incapable of causing serum-mediated hypersensitivity in both man and animals *(Capstick, P.B. et.al., 1970, Shenberg, E., and Torten, M., 1973, Torten, M. et.al., 1973 and Broughton, E.S. et.al., 1983)*. The use of leptospiral vaccines in man is, however, limited. Since there is little knowledge of the antigens that correlate with virulence and protective ability, vaccines should be made from serovars isolated in the regions they are to be used *(Torten, M., 1979)*. The fact that many domestic animals throughout the world become unrecognized carriers is probably the result of vaccination with locally inadequate vaccines produced elsewhere *(Anon, 1975)*.

Leptospirosis is a zoonosis with the widest geographical distribution.

It's continued existence is dependent upon the local presence of carrier animals and local environmental conditions that enable the organism's survival and maintenance outside the host. Because of the dynamic changes in the epidemiological conditions, only systematic surveillance can be expected to provide useful data on the factors involved in epidemiological cycles and on the need to introduce reasonable preventative measures. I have shown that cultural isolation is a better index of infection and carrier rates in wild animal surveys. This may probably be so for domestic animals. However, it is difficult to do and often unsuccessful. Although not much specific data is available on the outbreak patterns of leptospirosis, in at least one case a four to six year cycle has been observed in Israel *(Torten, M., et.al., 1970)*. It is possible that the same occurs elsewhere.

Surveillance for leptospirosis and for that matter any zoonosis can be developed at national level as a basis for international programmes. Only surveys conducted under adequate statistical control can yield reliable information about leptospirosis in any area and during any period *(Bull.Wld. Hlth.Org., 1972)*. Such programmes should go hand in hand with national development programmes; for example, in a country like Kenya where small ruminant projects are being developed and expanded in order to provide adequate and quick animal protein, sheep and goats should definitely be considered a special hazard to public health and adequate precaution taken. The role of the goat in the epidemiology of other diseases, e.g. Brucellosis is well known.

It is essential that countries that do not report or do not have a leptospirosis programme at all start seriously evaluating the public health significance and economic losses caused by human and domestic animal leptospiroses. Proper training is thus essential in equipping the public health personnel to diagnose and study the medical, veterinary and economic damages attributable to leptospirosis.

In conclusion, it appears that there are still several areas where further research may be conducted to evaluate the following problems:-

1. Despite the accepted fact that the microscopic agglutination test (MAT) is the reference serological test for leptospirosis, there is the need for a study to establish the standard medium for antigen preparation used in the test.
2. Research is essential for the fuller understanding of the pathogenesis and chemotherapy of leptospirosis.
3. The development and evaluation of serological tests, including tests to use with sera from domestic and wild animals.
4. The development of culture and transport media.
5. The epidemiology of leptospirosis: a comparison of the developing and developed countries.
6. The economic impacts of leptospirosis in both developing and developed countries.

ADDENDUM

ISOLATION OF A LEPTOSPIRE FROM A ONE WEEK DEAD COYPU

While writing up this project, it was decided that I should go back to Suffolk and Norfolk to prepare a video demonstration of the techniques of sampling, collection and processing of the specimens. Because of local adminstrative difficulties, it was not possible to obtain a live coypu and, therefore, one which was dead for one week but well preserved in a cold environment was used. This coypu was processed similarly to that described previously. The specimens obtained were treated as described previously, except that they were microscopically examined for the first time after 29 days.

After 29 days incubation, leptospires were seen in both urine and kidney cultures; first and second serial dilutions respectively. These were further serially diluted into media with 2% and 1% rabbit serum in media with 8-azaguanine (400µg/ml). *L.icterohaemorrhagie, L.hardjo, L.canicola* and *Patoc 1* were used as controls for the 8-azaguanine media. The orgaism did not grow in 8-azaguanine and was therefore pathogenic. A Kmety identification scheme could not be done in time for submission of this work.

Discussion

It was noticed during the opening of the animal that it was in a remarkably good physiological and anatomical state despite it being dead for one week. Therefore, it must have been well preserved by the weather during that time. On isolation of the leptospiral organism from the animal, a retrospective weather report for the previous week (March 1st to 6th, 1984) was obtained from the Huntingdon Meteorlogical Weather Station. This time period corresponds to the time the coypu had been dead before examination.

The week's temperature range was from 11.3^{o}C to -2.2^{o}C with an average of 6.6^{o}C (max) and 2.45^{o}C (min). The week's average humidity was 78.5% (max)

and 67% (min).

Apparently, under these conditions, there was very little autolysis, and the leptospires managed to survive for that period of time. Because of the asceptic technique used at the site of collection it is assumed that there is no possibility of casual contamination of leptospires from the water/soil. In addition, the organism was isolated from both the kidneys and urine.

TABLE XIII. Weather Report.

Day	Winds	Temperature readings		Humidity readings	
		Max.	Min.	Max.	Min.
1.3.84.	Westerly	9.9°C	3.7°C	97%	79%
2.3.84.	Westerly	4.6°C	2.2°C	85%	58%
3.3.84.	Westerly	6.1°C	1.5°C	94%	59%
4.3.84.	Variable	7.7°C	-2.2°C	96%	71%
5.3.84.	W.N.Westerly	11.3°C	6.1°C	99%	83%
6.3.84.	-	*11.3°C	3.4°C	*99%	52%

* the coypu was obtained before these figures were obtained.

Work done by Ellis, et.al. *(1982)* on abortions of cattle due to *L.hardjo* demonstrated the isolation of a leptospire from a 4 day dead foetus that was subject to 15°C for one day and 4°C for three days. It has been generally assumed that the autolysis which occurs after death rapidly kills the leptospires and renders them unculturable *(Smith, R.E., et.al. 1967, Ellis and Michna, 1976).*

Ellis's work and my isolation of a leptospire from a one week dead coypu that was well preserved probably demonstrates that it is possible to isolate leptospires from dead animals, only if they are well preserved enough to inhibit autolysis to progress fast. This may be useful in situations

where it is not possible to carry out cultural techniques outside the laboratory.

PUBLICATIONS ARISING FROM THIS PROJECT

1. Waitkins, S., and Wanyangu, S.W. (1984) *Leptospirosis: General Introduction.* Public Health Microbiological Digest (*in press*)

2. Wanyangu, S.W., Waitkins, S., and Palmer, M. *A survey of leptospirosis in the coypu (Myocastor coypus Molina) (In preparation)*

3. Wanyangu, S.W., Waitkins, S., and Palmer, M. *A comparison of the DIFCO and Patoc 1 slide antigens in the screening for leptospirosis. (in preparation).*

APPENDIX

APPENDIX I

SOME HOSTS FROM WHICH LEPTOSPIRES HAVE BEEN ISOLATED.

SCIENTIFIC NAME	COMMON NAME
	Man:
Homo sapiens	Man
	Domestic animals:
Bubalus bubalis	Buffalo
Felis domestica	Cat
Bos indicus & Bos taurus	Cattle
Canis familiaris	Dog
Copra hircus	Goat
Equus caballus	Horse
Mustela vison	Mink
Ovis arie arles	Sheep
Vulpes vulpes	Silver Fox
Sus domestica	Swine
	Wild animals:
Acomys wilsoni	Spiny mouse
Acthomys kaiseri	Bush rat
Akodon arviculoides	S. American field mouse
A. thaptomys nigrita	Field mouse
Alopex lagopus	Arctic Fox
Apodemus agrarius	Striped field mouse
A. flavicollis	Yellow-necked field mouse
A. microps	Field mouse
A. mystacinus	Field mouse

A. speciosus	Large Japanese field mouse
A. sylvaticus dichurus	Wood mouse, common field mouse
Ardea purpurea	Purple heron
Arvicanthis abissinicus	Ethiopian mouse
A. niloticus	Grass rat, Kusu
Arvicola terrestris amphibus	Water vole
A. terrestris	Water vole
Bandicota bengalensis	Black bandicoot rat
B. elliotana	Large bandicoot rat
B. gracilus	Mole rat
B. malabarica	Mole rat
B.indica nemorivaga	Taiwan bandicoot rat
Blarina brevicauda	Short-tailed shrew
Calleorscuirus erythraneus yobert	Squirrel species
Canis aureus	Asiatic jackal
C. lupus	Wolf
Capreolus capreolus	Roe deer
Castor canadensis	Beaver
Cavia aperea azarae	Cavy
C. pamparum	Cavy
C. aperea festina	Wild guinea pig
Cervus elaphus	Red deer
C. nippon	Japanese deer
Chaetophractus villosus	Hairy armadillo
Chilodonias nigra	Black fern (bird)
Chironectes minimus	Water opossum
Citellus citellus gelingeus	Suslik
C. pygmaeus	Little suslik
C. refescens erythrogenes	Squirrel

Clethrionomys glareolus	Bank vole
C. rutilus	Northern red-backed mouse
Conepatus semistriatus	Striped hog-nosed skunk
Cricetomys gambianus	African giant pouched rat
Cricetus cricetus	Common hamster
Crocidura sp.	White-toothed shrew
Cynopterus sp.	Short-headed fruit bat
Dama dama	Yellow deer
D. virginiana	White-tailed deer
Dasypus novemcinctus	Armadillo (nine-banded)
Dermacentor marginatus	Tick
Didelphis azarae	Opossum, Azara's opossum
D. marsupialis	Black-eared opossum
Dusicyon gymnocercus	Pampar fox
Echinolaelops echidninus	Spiny rat mite (China)
Erinaceus europaeus	Hedgehog
E. roumanicus transeoucosicus	Hedgehog
Felis bengalensis euptilura	Leopard
Gallinula chloropus chloropus	Moorhen
Hemiechinus auritus	Long-eared hedgehog
Herpestes auroautopunctatus	Lesser Indian mongoose
H. edwardsi	Mongoose species
H. ichneumon	Mongoose species
H. javanicus	Javan mongoose
H. urva	Crab-eating mongoose
Heterodon playtyrhinus	Hog-nosed snake
Heteromys desmarestianus	Spiny pocket mouse, Desmarest's
Hydromys chrysogastor	Australian water rat, Eastern.
Isoodon macrourus	Large northern short-nosed Bandicoot

Ixobrychus minitus	Little bittern
Lacerta agilis	Lizard
Lemniscomys straiatus	Spotted grass mouse
Lupus europaeus	Brown hare
L.griselda	Single-striped grass mouse
Liomys adspersus	Panama spiny pocket mouse
Lagomorpha	Porcupine
Lynx rufus	Bobcat
Macaca sylvana	Barbary ape
Marmota monax	Woodchuck
Mastomys nataliensis	Multimammate rat
Melomys cervinipes	Fawn-footed, naked-tailed rat
M.littoralis	Smaller naked-tailed rat
Mephitis mephitis	Striped skunk
Meriones tamariscinus	Tamarisk gerbil
Micromys minutus soricinus	Harvest mouse
Microtus agrestis hirtus	Vole
M.arvalis	Common field vole
M.fortis pelliceus	Reed vole
M.gregalis	Narrow-skulled vole
M.quentheri	Gunther's vole
M.majozi	Vole species
M.montebelloi	Vole species
M.oeconomus	Root vole
M.oeconomus ratticeps	Root vole
M.pennsylvanicus	Meadow vole
Mus musculus	House mouse
M.brevirostris	House mouse
M.hortulanus	House mouse

M.lustanicus	House mouse
M.molessinus	House mouse
M.spicilegus	Gleaner mouse
M.spretus	House mouse
Mustela erminea	Stoat, ermine
M.nicalis	Weasel
M.putorius	European polecat
Myocastor coypus	Nutria, coypu
Myotis sp	Mouse-eared rat
Nectomys squamipes	Water rat, S.American
Neomys fodiens	European water shrew
Odocoileus viginiana	White-tailed deer
Ondatra zibethecus	Muskrat
Ornithodorus tartakovskyi	Tick (argasid)
Oryctolagus cuniculus	European rabbit
Oryzomys caliginosus	Dusky rice rat
O.eliuris	Rice rat
O.nigripes	Rat species
O.ratticeps	Rice rat
Osymycterus quaestor	Burrowing mouse
Otomys angoniensis	Swamp rat
Paguma larvata taivana	Masked-palm civet
Pan troglodytes	Chimpanzee
Panthera tigris	Tiger
Paradoxurus hermaphroditus	Palm civet
Paramelis nasuta	Long-nosed bandicoot
Peromyscus sp.	Deer mouse or white-footed mouse
P.maniculatus	Deer mouse
P.polionotus	Old field mouse
Philander opossum	Four-eyed opossum

Pitymys subterraneus majori	Borrowing vole, pine vole
Procyon lotor	Raccoon
Proechimys semispinosus	Spiny rat
Pseudmys sp.	Turtle species
Rana pipiens	Frog
Rattus assimilis	Allied rat
R.assimilis fuscipes	Southern bush rat
R.bartelsii	Bartel's rat
R.bowersi	Bower's rat
R.conatus	Canfield's rat, dusky field rat
R.culmorum	Australian house rat
R.exulans	Little rat, pacific rat
R.exulans concolor	Little rat, pacific rat
R.decumanus	Norway rat species
R.mulleri	Muller's rat
R.norvegicus	Norway rat, brown rat
R.rajah	Rajah spiny-backed rat
R.rattus	Roof or black rat
R.argentiventer	Ricefield rat, house rat
R.brevicaudatus	House rat (Java)
R.diardi	House rat (Malaysia)
R.flavipectus	House rat (China)
R.jalorensis	Malaysian field rat, house rat
R.mindanensis	Rat species
R.rapheus	Rat species (or mouse)
R.sabanus	Large spiny-backed rat, noisy rat
R.whitehaedi	Whitehead's rat (Borneo)
Reithrodontomys megalotis	Western harvest mouse
Rhipicephalus sanguineus	Brown dog tick

Rhombomys opimus	Great gerbil
Saccostomys campestris	African pouched rat
Sciata betulina corossont	Northern birch mouse
Sciurus niger	Fox squirrel
Sigmodon hispidus	Cotton rat
Sorex araneus	Common shrew
S.minutus	Pygmy shrew, lesser shrew
Spilogale putorius	Spotted skunk
Suncus caevleus gigantous	Shrew species
S.luboriecus	Shrew species
S.murinus	Musk shrew
Sus scrofa	Wild boar
Sylvilagus floridanus	Eastern cotton-tailed rabbit
S.paladilagus	Rabbit species
Tetera robusta vicina	Gerbil
Talpa europaea altaica	Common mole
Thylacis obesulus	Kangaroo rat
Trichosurus sp.	Australian opossum
Tylomys panamensis	Panama climbing rat
Urocyon cinereoargenteus	Grey fox
Uromys caudimaculatus	Giant naked-tailed rat
Vulpes fulva	Red fox
Zayophus californsonus	California sea lion
Zygodontomys basiurus	Cane rat
Z.cherrie	Cherrie's rat

A P P E N D I X: II

(A) MATERIALS REQUIRED FOR FIELD PROJECT.

(i) Experimental animals: the coypu *(Myocastor coypus* Molina).

(ii) Traps and bait: cage traps with carrots or apples as bait.

(B) MEDIA AND SOLUTIONS.

Solutions.

1. Phosphate buffer solution (PBS)

Na_2HPO_4	16.6g
KH_2PO_4	2.172g.

Dissolve in 1 litre of distilled water.

2. "Salts solution"

NaCl	38.5g
NH_4Cl	5.35g.
$MgCl_2.6H_2O$	3.81g

Dissolve in 1 litre of distilled water.

3. Other salts

$CuSO_4.5H_2O$	30mg + 100ml distilled water
$FeSO_4.7H_2O$	500mg + 200ml " "
$ZnSO_4.7H_2O$	80mg + 200ml " "

4. Vitamins and amino acids

Vit. B_{12}	10mg + 100ml distilled water
Vit. B_1	200mg + 100ml " "
L-Cystine	

5. Tween 80 (BDH)

6. Distilled water

7. Bovine albumin

Dissolve 50g. Pentax albumin, fraction V in 1030ml boiled distilled water and add 40ml of phosphate buffer.

8. Rabbit serum

9. Agar

10. 5-Fluorouracil (5-FU) (Sigma F6627)

1g. 5-FU + 50ml distilled water + 2ml 2NaOH. Heat gently (less than $56^{o}C$) for 1 - 2 hours and adjust the pH to 7.4 - 7.6.

Media.

(a) Ellinghausen McCullough media with 2% rabbit serum and 0.1% agar

Prepare as follows:

1. Put 2.8 litres of distilled water in a 5 litre flask.
2. Add 160ml of phosphate buffer solution.
3. Add 200ml of "Salts solution".
4. Add 4ml of copper sulphate solution.
5. Add 40ml of zinc sulphate solution.
6. Add 80ml of freshly prepared ferrous sulphate solution and shake for five minutes.
7. Add 0.8g of L-Cystine and shake for one minute.
8. Filter through triple thickness of Whatman No. 1 filter paper.
9. Add 4.8ml Tween 80 pre-warmed at $56^{o}C$.
10. Add 236ml distilled water.
11. Autoclave at 15 lbs. for 15 minutes.
12. Add 8ml Vit. B_{12} and 4ml Vit. B_{1} solutions.
13. Add 2% (100ml) rabbit serum inactivated at $56^{o}C$ for 30 minutes to bovine albumen solution, filter through Whatman No. 1 filter paper and add to the sterilized base medium (above).
14. Adjust the pH to 7.3 - 7.4.
15. Sterlize by filtering through a sandwich of millipore membranes (0.22μ, 0.45μ and 1.2μ) into sterile bottles.
16. Add 100ml 1% agar to every litre of medium.
17. Incubate bottles at $37^{o}C$ to test for sterility.

(b) Ellinghausen McCullough media with 2% rabbit serum, 0.1% agar and 0.02% 5-FU (200µg/ml)

1. Prepare as above.

2. Add 20ml of 5-FU solution to 1 litre of medium.

A P P E N D I X: III

Shows sex, approximate age in days, weight in Kg and location where coypu were collected

Coypu	Date Collected	Sex	Approximate weight	Approximate age in days	Location found	Any other feature
1.	15/11	F	1 Kg	46	GR. TM 49 on bank of River Waveney in Burgh St. Peter's.	Marsh and grazeland. Dairy cattle kept on pastures. A lot of Willow-herb & Dock.
2.	16/11	M	5 Kg	317	GR. TM 49. on a dyke in North Cove	" " " " "
3.	16/11	F	2 Kg	113	" " "	" " " " " Was pregnant with 5 fetuses
4.	16/11	M	3 Kg	310	GR. TM 49 in Barnby Parish on a dyke	Grazeland and dairy cattle
5.	16/11	M	3 Kg	201	" " "	" " " " "
6.	16/11	M	1 Kg	n.d	" " "	" " " " "
7.	16/11	F	2 Kg	254	" " "	" " " " "
8.	16/11	M	7 Kg	449	GR. TM 49 on bank of River Waveney in Burgh St. Peter's.	Marsh and grazeland. Dairy cattle kept on pastures. A lot of Willow-herb & Dock.
9.	16/11	F	7 Kg	388	" " "	" " " " " Pregnant with 5 fetuses

Coypu	Date Collected	Sex	Approximate weight	Approximate age in days
10.	17/11	M	6 Kg	450
11.	17/11	M	5 Kg	117
12.	10/1	F	7 Kg	735
13.	10/1	M	4 Kg	1333
14.	10/1	M	1 Kg	77
15.	10/1	M	3 Kg	352
16.	10/1	F	1 Kg	63
17.	10/1	F	1 Kg	63
18.	10/1	M	1 Kg	77
19.	10/1	N.K	1 Kg	86
20.	11/1	M	2 Kg	172
21.	11/1	M	1 Kg	104
22.	11/1	F	2 Kg	188
23.	11/1	M	2 Kg	158
24.	11/1	M	4 Kg	188
25.	12/1	F	3 Kg	167
26.	12/1	F	8 Kg	553

Location found	Any other feature
GR. TM 13 on a trout lake, Tattingstone Parish.	Horses on farm
GR. TM 13 on a stream	Apple orchard and grazeland
GR. TG 40 on Haddiscoe "island" on the banks of the River Waveney	Pasture in Summer. Was pregnant with 7 fetuses.
" " " "	" " " " "
" " " "	" " " " "
" " " "	" " " " "
" " " "	" " " " "
" " " "	" " " " "
" " " "	" " " " "
" " " "	" " " " "
GR. TG 40 Fritton Parish	" " " " "
" " " "	" " " " "
" " " "	" " " " "
	Pregnant with 1 fetus.
" " " "	Pasture in Summer
" " " "	" " "
GR. TM 49. Herring Fleet	" " "
GR. TM 49. Somerleyton	" " "
	Pregnant with 6 fetuses.

Coypu	*Date Collected*	*Sex*	*Approximate weight*	*Approximate age in days*
27.	13/1	F	3 Kg	559
28.	13/1	F	4 Kg	531
29.	13/1	M	4 Kg	680

GR.TG = Grid Reference Number

n.d = not done

N.K = Not Known

Location found	Any other feature
GR. TG 40 Haddiscoe "island"	Pasture in Summer. Pregnant with 5 fetuses. Jaundiced liver.
" " " "	Pregnant with 11 fetuses.
" " " "	Pasture in Summer

A P P E N D I X: IV

METHOD FOR SLIDE AGGLUTINATION TEST

Materials required for the test

Glass slides 150mm X 75mm X 2mm engraved with 3 rows of squares (25mm X 25mm)

"50 Droppers" delivering 0.02ml (units volume for test)

Rotating apparatus

Patoc Slide Test Antigen

P.B.S. (pH 7.2) ..... Oxoid BR 14a

1% Azide Saline

Slide agglutination test antigen preparation

1. Inoculate 1,000ml Ellinghausen and McCullough media with *Leptospira (Biflexa) Semaranga serovar Patoc 1* and incubate at 30°C in a shaking incubator for 7 days.
2. Check the culture for quality of growth and presence of contaminating bacteria by both dark field microscopy and plating on blood agar at 37°C. Kill the culture by adding neutralized formalin to give a final concentration of 0.2% and leave for 30 minutes.
3. Centrifuge at 16,000 G at +5°C for 30 minutes and re-suspend the deposit in 1% azide saline.
4. Re-centrifuge the culture and re-suspend the deposit in about 50.0mls of 1% azide saline and boil in a water bath for 30 minutes. Repeat this procedure twice finally re-suspending the deposit in about 10.0mls azide saline and store at +4°C.
5. Dilute the antigen for use with 1% azide saline to give a fairly dense suspension equivalent to McFarland 20. McFarland standards are prepared as follows:-
   a) SOLUTION A ..... 1% solution of Barium Chloride
   b) SOLUTION B ..... 1% Sulphuric Acid

c) Prepare the "McFarland 20" standard by mixing as follows:-

2.0 ml of solution A and 8.0 ml of solution B (0.1 ml solution A made up to 10.0 ml with solution B will give McFarland 1. 0.2 ml solution A made up to 10.0 ml with solution B will give McFarland 2 etc.)

The quality of the antigen is checked against PBS and known positive and negative sera. (N.B., if the antigen is granular in PBS centrifuge FOR 5 MINUTES at 1,000 rpm and retain the supernatant).

Performance of the test.

1. Sample test serum as follows:-
   a) Put 9 volumes PBS into a 3" by ½" test tube.
   b) Put one volume of test serum onto an engraved slide and one volume into the 9 volumes of PBS (giving a 1/10 dilution of serum).
2. Mix the 1/10 dilution and put one volume onto the engraved slide.
3. Put one volume of shaken Patoc Slide Antigen onto each drop of serum on the slide.
4. Mix with an applicator stick spreading to a diameter approximately 15 mm.
5. Rotate at 125 rpm for 4 minutes.
6. Read immediately using an oblique light source against black background.
7. Grade and record the agglutination as follows:-

No agglutination	NEGATIVE
Very weak, fine agglutination	+
Particulate agglutination	+1
Small aggregates and turbidity	+2
Large aggregates and slight turbidity	+3
Large aggregates and no turbidity	+4

The positive control serum should give +4 reaction neat and at 1/10.

8. Discard the used glass slides into disinfectant.

APPENDIX V

Method for Complement Fixation Test

Reagents and apparatus required:

CFT Diluent	Oxoid BR 16
Complement (preserved guinea pig serum)..	Wellcome CT01
Rabbit Haemolytic serum (H.I.B)	Wellcome VD15
Sheep cells	Tissue Culture Services
CFT Antigen	Leptospira Reference Unit
Microtitre Trays "U"	Sterilin M24.A.

CFT compound antigen preparation

Seven day old cultures of *L.Semaranga serovar patoc 1*, *L.Hebdomadis serovar jules*, *L.Autumnalis serovar bulgarica*, *L.Hebdomadis serovar hardjo* (now named *L.Wolffii serovar hardjo*), *L Canicola serovar canicola*, and *L. Wolffii serovar wolffii* were grown in Ellinghausen McCullough media containing 1% (v/v) of pooled normal rabbit serum, free from leptospiral agglutinins and previously heat inactivated (56°C 30 min). The antigen was then prepared as follows:

1. Inoculate 20 ml of seven day old culture in 1 litre of Ellinghausen McCullough media (1 litre was used for each culture) and incubate at 30°C in a shaking incubator for seven days.
2. Kill the cultures by adding Thiomersol (BDH) to give a final concentration of 1 in 10,000 (2% w/v solution of Thiomersol (BDH) is prepared using distilled water. 5 ml of this solution when added to 1 litre of culture gives the above concentration).
3. Check the cultures for contaminants, then centrifuge at 23,000 G at 10°C for 30 minutes. Repeat three times, resuspending the deposit in about 10 ml 0.1% sodium azide and finally in 25 ml of 0.1% sodium azide.

4. Store the final suspension at 4 - 5°C for two weeks in a glass bottle.

5. Make the suspension up to 50 ml with 0.1% sodium azide and acid-heat treat to remove the anticomplementary effect. This is done as follows: One volume of 0.4 M HCl is added to 19 volumes of antigen and mixed immediately. The mixture is heated in boiling water bath for 15 minutes, cooled and mixed thoroughly. Adjust the pH to 7.2 by use of NaOH.

6. Assay the antigen as follows:

   (a) Titrate each antigen in presence of complement. This is necessary because the antigen is usually slightly anticomplementary and also aids to determine the complement titre for each antigen dilution (HC_{50})

   (b) Titrate each antigen against it's homologous antiserum using the requisite strength of complement (2 HC_{50}) for each antigen dilution as determined in Step (a). The optimum dilution is that which shows the highest titre of the antiserum. The reciprocal of this dilution when multiplied by the volume available, gives the "effective use volume". When all antigens are assayed, mix them as shown in the example below:-

Name	Available volume	Use dilution	Effective-use volume	Ratios of use-dilutions	Amounts to be taken
PATOC 1	50ml	64	3200ml	1	50ml
JULES	50ml	64	3200ml	1	50ml
BULGARICA	50ml	64	3200ml	1	50ml
HARDJO (Cow 204)	50ml	128	6400ml	0.5	25ml
CANICOLA	50ml	64	3200ml	1	50ml
WOLFFII	50ml	128	6400ml	0.5	25ml

A series of dilutions of the compound antigen is made and titrated against all sera used previously and the dilution that gives the optimum titre is used. Store the antigen at $4^{o}C$ (N.B., if the antigen is excessively anticomplementary, it may be re-acid treated before storage).

Performance of Test

1. Make a 1:10 dilution of the patient's/Test serum (0.05 ml serum + 0.45 ml diluent). Also include a Patoc serum as a positive control.
2. Inactivate the 1:10 dilution in a $56^{o}C$ water bath for 30 minutes.
3. Starting at Row B put one volume of diluent into all the wells of two columns for each serum to be tested. The odd numbered columns will have antigen (test column) and the even numbered column will be the control to detect anticomplementary activity.
4. Put one volume of inactivated serum dilution into the first two wells of the test and control columns.
5. Pre-wet a Dynatech microdiluter with diluent and then double dilute from Row B. Mix twenty times for each dilution. Sterilize the diluters by heating in a bunsen flame.
6. Put one volume of diluent into all the wells of even numbered columns.
7. Make complement up to it's working dilution ($2HC_{50}$). (See the end of these instructions for method of preparing the complement). Keep the working dilution of complement at $+4^{o}C$ if it is not required immediately.
8. Put one volume of diluted complement into all the wells.
9. Put one volume of leptospiral CFT antigen, diluted to it's working strength, into the wells of the odd numbered (test) columns.
10. Set up a complement control at $2HC_{50}$, $1HC_{50}$, $\frac{1}{2}HC_{50}$, $\frac{1}{4}HC_{50}$, all with and without antigen. These controls are performed in duplicates as follows:-
    (a) Put one volume of CFT diluent into each well of two of four wells for the complement + antigen control and also put two volumes

of buffer into each well of two rows of four wells for the complement control without antigen

(b) Dilute the prepared complement in 3" by ½" tubes as follows:-

	$2HC_{50}$	$1HC_{50}$	$\frac{1}{2}HC_{50}$	$\frac{1}{4}HC_{50}$
Diluent	-	0.5ml	1.5ml	3.5ml
$2HC_{50}$ complement	0.5ml	0.5ml	0.5ml	0.5ml

These dilutions must be mixed thoroughly.

(c) Put one volume of each complement dilution into the wells of the appropriate columns.

(d) Add one volume of antigen to all wells of the appropriate control rows.

11. Shake the plates on a shaker.
12. Put the plates at $+4^{o}C$ overnight.
13. Wash the sheep cells in readiness for the following day (see end of these instructions for the method).

Next day continue as follows:

14. Sensitize the sheep cells by incubating the cells/H.I.B. mixture in a $37^{o}C$ incubator for 30 minutes (see end of these instructions for the method of preparing sheep cells). Also remove the plates from the refrigerator and put them in the $37^{o}C$ incubator for 30 minutes.
15. Put one volume of the sensitized sheep cells into all the wells.
16. Shake the plates to mix.
17. Incubate at $37^{o}C$ for 15 minutes.
18. Shake the plates and return them to the incubator for a further 15 minutes.
19. Shake the plates and put them at $+4^{o}C$ to allow the red cells to settle.
20. Read results:

(a) The end point is the highest dilution of serum which shows at least 50% fixation.

(b) The control column should show complete lysis. The presence of red cells indicate anticomplementary activity.

(c) The complement controls should show lysis at $2HC_{50}$ and 50% lysis at $1HC_{50}$.

Preparation of Reagents

Complement

(a) Reconstitute with 2.0 mls of distilled water. Prepare fresh each week.

(b) On day of test: Dilute ONE VOLUME of the reconstituted complement with SEVEN VOLUMES of DISTILLED WATER giving a 1/10 dilution of complement.

Further dilution to the working strength of $2HC_{50}$ is done in DILUENT (for example, a working dilution of 1/30 is prepared by adding 4.8 ml diluent to 2.4 ml of the 1/10 dilution of complement). The titre ($1HC_{50}$) is pre-determined by titrating the complement in the presence of antigen.

Sheep Cells

(a) Wash the cells three times in diluent (centrifuge at 2000 rpm for 5 minutes).

(b) Re-suspend the cells in twice their volume of diluent and mix thoroughly.

(c) Measure the cell concentration with a spectrophotometer or haematocrit. The concentration will be about 20%.

(d) Store the prepared cells at $+4^{o}C$ overnight.

Sensitization of Sheep Cells

(a) Prepare 10.0 ml of 4% sheep cells in diluent.

(b) Prepare 10.0 ml of the haemolytic serum at it's working strength, the titre having been previously determined by performing a complement/haemolytic serum; chessboard titration.

(c) Mix thoroughly by pouring from beaker. Continue from Step 14 above.

APPENDIX VI

Method for Microscopic Agglutination Test (MAT.

Reagents and apparatus required.

Phosphate buffered saline (PBS) pH 7.2 Oxoid BR 14a.

Antigen ..... Formalised leptospiral cultures

Membrane filters. 0.45µm.

Syringes. 5.0 ml.

Perspex agglutination trays.

Glass slides, 125mm by 75mm: 1.0mm thick ... Solmedia

Formalin pH 7.2

Preparation of antigens

1. Select leptospiral strains to represent the following serogroups:

i.	*Icterohaemorrhagiae*	*xi.*	*Sejroe*
ii.	*Javanica*	*xii.*	*Saxkoebing*
iii.	*Celledoni*	*xiii.*	*Wolffii*
iv.	*Canicola*	*xiv.*	*Mini*
v.	*Pyrogenes*	*xv.*	*Bataviae*
vi.	*Ballum*	*xvi.*	*Tarassovi*
vii.	*Autumnalis*	*xvii.*	*Pomona*
viii.	*Australis*	*xviii*	*Cynopteri*
ix.	*Grippotyphosa*	*xix.*	*Semaranga.*
x.	*Hebdomadis*		

2. Grow the selected strains in 20.0 ml of Ellinghausen and McCullough leptospiral culture medium incubated at 27°C for 7 days.
3. Check the cultures for quality of growth and presence of contaminating bacteria by viewing with dark field microscopy and plating out on blood agar incubated at 37°C.
4. Kill the cultures by adding neutralized formalin to give a final concentration of 0.2%.
5. Pool two or three representative serotypes from each of the 19 serogroups to form the antigen pools required for the test.

6. Perform a pool check on new pools and subsequently once a week (see end for method).

N.B. In this project the following antigens were used:

Antigen pools for Microscopic Agglutination Test (MAT)

	Serogroup	Pools
I.	*Icterohaemorrhagiae*	*RGA, Wijnberg, Mankarso*
II.	*Javanica*	*Javanica*
III.	*Celledoni*	*Whitcombi*
IV.	*Canicola*	*Canicola, Schueffneri*
V.	*Pyrogenes*	*Pyrogenes, Robinsoni, Biggis*
VI.	*Ballum*	*Mus 127, Castellonis*
VII.	*Autumnalis*	*Rachmati, A.akiyami*
VIII,	*Australis*	*Ballico, Lora, Jalna*
IX.	*Grippotyphosa*	*CH 31, Moskova V.*
X.	*Hebdomadis hebdomadis*	*Hebdomadis, Kambale*
XI.	*Hebdomadis sejroe*	*Sejroe*
XII.	*Hebdomadis saxkoebing*	*Ricardi, Saxkoebing*
XIII.	*Wolffii wolffii*	*Wolffii, Hardjo (cow 204)*
XIV.	*Hebdomadis mini*	*Mini, Georgia*
XV.	*Bataviae*	*Poidjan, Djatzi*
XVI.	*Tarassovi*	*Panama, G 129*
XVII.	*Pomona*	*Mitis, Perepelicin, Rama LT 955*
XVIII.	*Cynopteri*	*Putembo*
XIX.	*Semaranga*	*Patoc*

Performance of test

1. Prepare a 1/100 dilution of test serum. (N.B. it may be necessary to filter the serum through a 0.45 micron filter. In my project this was not done as the serum samples were quite limited in amounts).
2. Put one volume of test serum dilution into each of 19 wells of a perspex agglutination tray, one row for each dilution. Use two adjacent plates if the test serum has been titrated.
3. Put one volume of PBS into a row of 19 wells for a negative antigen control.

4. Add one volume of each antigen pool to all the wells of it's respective column of the perspex agglutination tray(s).
5. Tap the trays to mix.
6. Put the trays in a moist chamber.
7. React overnight at room temperature.
8. Read agglutination using dark field microscopy as follows:
   a) Use a large bore pipette to mix gently the contents of a well and transfer a drop to a glass tile. Rinse the pipette between columns and sample from the highest dilution of titrations.
   b) Grade the degree of agglutination as follows:-

+	Complete
(+)	60% - 90%
±	50%
(±)	20% - 40%
tr	10% - 20%
-	No Agglutination

9. Immerse glass slides and agglutination trays in 2.5% Sterite and leave overnight before washing.

Pool Check

1. Use the following reference antisera to perform pool check.

Pool	Serogroup	Check serum
i.	*Icterohaemorrhagiae*	*RGA*
ii	*Javanica*	*Veldrat Batavia 46*
iii.	*Celledoni*	*Celledoni*
iv.	*Canicola*	*Vleermuis 90 C*
v.	*Pyrogenes*	*Robinsoni*
vi.	*Ballum*	*Mus 127*
vii.	*Autumnalis*	*Bankinang 1*
viii.	*Australis*	*Lora*
ix.	*Grippotyphosa*	*Moskva V*
x.	*Hebdomadis*	*Hebdomadis*
xi.	*Sejroe*	*M 84*

xii.	*Saxkoebing*	*Mus 24*
xiii.	*Wolffii*	*Hardjoprajtino*
xiv.	*Mini*	*Szwajizak*
xv.	*Bataviae*	*Van Tienen*
xvi.	*Tarassovi*	*Mitis Johnson*
xvii.	*Pomona*	*Pomona*
xviii.	*Cynopteri*	*3522 C*
xix.	*Semaranga*	*Patoc*

2. Dilute the sera to 1/160 as follows:

   a) Put 4.0 ml PBS into a bijou.

   b) Add 25 µl reference serum.

   (The final dilution after the addition of antigen will be 1/320).

   Store at +4^{o}C, discard after 2 weeks.

3. Put one volume of each diluted reference serum into one well of an agglutination tray (this will be a total of 19 wells).

4. Put one volume of PBS into an equivalent number of wells, parallel to those containing the reference sera to act as negative controls.

5. Put one volume of each antigen pool into the well containing it's respective group antisera and a well containing PBS.

6. Tap the trays to mix and put in a moist chamber.

7. React overnight at room temperature.

8. Read the test using dark field microscopy as in Step 8 of "Performance of Test".

   An antigen pool is considered satisfactory if there is:

   a) At least 50% agglutination with the homologous antiserum.

   b) No clumping in PBS.

9. Immerse glass slides and agglutination trays in 2.5% Sterite after use and leave overnight before washing.

APPENDIX VII

Warthin-Faulkner method for staining paraffin fixed sections for spirochaetes

Fixation

Formal saline should be employed for fixation.

Special Reagent required

Stock gelatin (for use with developer solution).

Gelatin .... 5 g

Walpole's sodium acetate-acetic acid buffer (pH 3.6) ..... 100 ml

Add 1 ml of 1: 10,000 merthiolate to prevent the growth of moulds.

Method

1. Bring paraffin sections to water.
2. Wash in 1:25 dilution of stock pH 3.6 acetate-acetic acid buffer for 5 minutes
3. Impregnate with 1% silver nitrate in dilute pH 3.6 buffer (as in 2) for 45 minutes at 60°C.
4. While sections are in silver nitrate, prepare the following developer solution. Mix 15 ml of the stock gelatin solution and 3 ml of 2% silver nitrate in pH 3.6 acetate-acetic acid buffer, both previously heated to 60°C. Add 1 ml of fresh 3% hydroquinone in pH 3.6 acetate-acetic acid buffer. Mix and use immediately.
5. Place slides on staining rack and flood with freshly prepared developer. When sections become brown to grey-yellow, and the developer turns brownish-black, rinse with warm water (55-60°C) and then with distilled water.
6. Dehydrate, clear and mount in Canada balsam or D.P.X.

Results

Spirochaetes ..... Black

Tissue elements .....Shades of yellow

Note: Over-development will give precision and thickened black spirochaetes, and should be overcome by carrying through 2 or 3 sections with varying times of developments.

Sections may be toned in gold chloride, and counter-stained if desired.

Automatic staining technique for haematoxylin and eosin (HE)

1. Put the slides in an oven at 60°C for 30 minutes.
2. Cool the slides.
3. Put in the machine which treats them as follows:

a)	TCF III	3 minutes
b)	Absolute alcohol I	3 minutes
c)	70% alcohol	3 minutes
d)	Distilled water	3 minutes
e)	Harn's haematoxylin	10 minutes
f)	Distilled water	2 minutes
g)	0.5% acid alcohol	15 seconds
h)	Distilled water	2 minutes
i)	PBS pH 7.8	5 minutes
j)	PBS pH 7.8	5 minutes
k)	Distilled water	90 seconds
l)	0.2 eosin	5 minutes
m)	Distilled water	1 minute
n)	Absolute alcohol (3 times)	2 minutes
o)	TCF III	3 minutes

Transfer sections to final bath of TCF prior to mounting.

APPENDIX VIII

Method for Difco Slide Agglutination Test

1. Place 0.01 ml of serum to be tested in the appropriate number of squares on a slide or ruled plate with 0.2 ml pipette.
2. Using supplied dropper, add 1 drop of each desired antigen pool to separate drop of serum.
3. Using a separate piece of applicator stick, mix each drop of antigen and serum.
4. Rotate the plate by hand approximately 5 - 10 times.
5. Place on the mechanical rotator for 4 minutes at 125 rpm.
6. Observe reaction over light.
7. Record agglutination as follows:-

Positive	+	definite clumping
Doubtful	$\pm$	slight clumping
Negative	-	even suspension.

APPENDIX IX

Results of comparison of M.A.T., Patoc and Difco Tests on Coypu sera

Coypu Serum	Patoc Neat	(SAT) 1:10	Result	DIFCO 1	2	3	(SAT) 4	5	6	Result
1	-	-	N	-	-	-	-	-	-	N
2	-	-	N	-	-	-	-	-	-	N
3	-	-	N	(±)	-	-	-	-	-	D
4	-	-	N	-	-	-	-	-	-	N
5	+1	-	D	+	-	(±)	-	-	-	P
6	-	-	N	-	-	-	-	-	-	N
7	-	-	N	nd	nd	nd	nd	nd	nd	-
8	-	-	N	-	-	-	-	-	-	N
9	tr	-	N	-	-	-	-	-	-	N
10	+1	-	D	-	-	-	-	-	-	N
11	-	-	N	-	-	-	-	-	-	N
12	-	-	N	-	-	-	-	-	-	N
13	tr	-	N	-	-	-	-	-	-	N
14	-	-	N	-	-	-	-	-	-	N
15	+1	-	D	+	(±)	+	+	+	+	P
16	-	-	N	-	-	-	-	-	-	N
17	-	-	N	-	-	-	-	-	-	N
18	-	-	N	-	-	-	-	-	-	N
19	-	-	N	nd	nd	nd	nd	nd	nd	-
20	-	-	N	-	-	-	-	-	-	N
21	-	-	N	-	-	-	-	-	-	N
22	tr	-	N	(±)	-	-	-	-	-	D
23	+1	-	D	-	-	-	-	-	-	N
24	-	-	N	-	-	-	-	-	-	N
25	-	-	N	nd	nd	nd	nd	nd	nd	-

Results of comparison of M.A.T., Patoc and Difco Tests on Coypu sera

Coypu Serum	Patoc	(SAT) 1:10	Result	DIFCO (SAT 1	2	3	4	5	6	Result
26	tr	-	N	-	-	-	-	-	-	N
27	+2	-	D	-	-	-	-	-	-	N
28	+2	-	D	(±)	-	-	-	-	-	D
29	+1	-	D	(±)	-	-	-	-	-	D

N = Negative, D = Doubtful, P = Positive, nd = not done, tr = trace.

+1 = Particulate agglutination, +2 = Small aggregates and turbidity, +3 = Large aggregates and slight turbidity, +4 = Large aggregates and no turbidity, + = Positive, ± = Doubtful, - = Negative.

M.A.T. Results on Coypu sera

Coypu No.	*Ict.*	*Bal.*	*Aut.*	*Aus.*	*Sej.*	*Sax.*	*Min.*	*Bat.*
2	160							
3	320							
5	640		320	80				
7	320							
9	160							
15		160			1280	80	80	160

Results of comparison of M.A.T., Patoc and Difco Tests on Human sera

Human Serum	Patoc Neat	(SAT) 1:10	Result	DIFCO 1	2	3	(SAT) 4	5	6	Result
1991/83	+2	-	D	+	+	+	-	+	+	P
2093/83	+2	-	D	+	-	-	(±)	(±)	-	P
2179/83	+1	-	D	-	-	-	-	-	-	N
2263/83	+3	+2	P	+	(±)	+	+	+	(±)	P
2416/83	-	-	N	-	-	-	-	-	-	N
2426/83	+4	+4	P	+	+	+	+	+	+	P
2429/83	+3	tr	P	(±)	-	+	+	(±)	-	P
2440/83	+1	-	D	-	-	-	-	-	-	N
16/84	+1	-	D	-	-	-	-	-	-	N
18/84	+2	-	D	-	-	-	-	-	-	N
25/84	-	-	N	-	-	-	-	-	-	N
26/84	-	-	N	-	-	-	-	-	-	N
35/84	-	-	N	-	-	-	-	-	-	N
36/84	+4	tr	P	(±)	-	(±)	+	(±)	-	P
37/84	+2	tr	D	+	-	+	(±)	(±)	-	
38/84	+4	+4	P	+	+	+	+	+	+	P
40/84	-	-	N	-	-	-	-	-	-	N
42/84	-	-	N	-	-	-	-	-	-	N
43/84	+2	tr	D	-	-	-	-	-	-	N
48/84	-	-	N	-	-	-	-	-	-	N
54/84	+2	-	D	-	-	-	-	-	-	N
59/84	+2	-	D	-	-	-	-	-	-	N
60/84	+1	-	D	-	-	-	-	-	-	N
61/84	tr	-	N	-	-	-	-	-	-	N
62/84	tr	-	N	-	-	-	-	-	-	N
63/84	+1	-	D	-	-	-	-	-	-	N

Human Serum	Patoc Neat	(SAT) 1:10	Result	DIFCO 1	2	3	(SAT) 4	5	6	Result
64/84	+1	-	D	-	-	-	(±)	(±)	-	D
70/84	-	-	N	-	-	-	-	-	-	N
72/84	tr	-	N	-	-	-	-	-	-	N
78/84	+4	+4	P	+	+	+	+	+	+	P
80/84	-	-	N	-	-	-	-	-	-	N
81/84	-	-	N	-	-	-	-	-	-	N
84/84	+4	+4	P	+	+	+	+	(±)	-	P
87/84	-	-	N	-	-	-	-	-	-	N
88/84	+1	-	D	-	-	-	-	-	-	N
93/84	+1	-	D	-	-	-	-	-	-	N
96/84	+2	-	D	(±)	-	(±)	-	-	-	D
97/84	+1	-	D	-	-	-	-	-	-	D
103/84	+4	+4	P	+	+	+	+	+	+	P

N = Negative, D = Doubtful, P = Positive, tr = trace.

+1 = Particulate agglutination, +2 = Small aggregates and turbidity, +3 = Large aggregates and slight turbidity, +4 = Large aggregates and no turbidity, + = Positive, ± = doubtful, - = Negative.

M.A.T. Results on Human sera

(Titre expressed as reciprocal)

Serogroups	Serum number										
	1991	2093	2263	2426	2429	36	38	78	84	96	103
Ictero				5120			80	320			5120
Javanica				40							
Celledoni					80				40		
Canicola	40	80		80			80	160	40		640
Pyrogenes				40			40				80
Ballum				40							320
Autumnalis				40							5120
Australis											80
Grippotyphosa	40										40
Hebdomadis			40		160						
Sejroe		160	40	320	640	640	640	1280	640	640	640
Saxkoebing		40			320	40	80		40		40
Wolffii	40	40		160	5120	5120		640	640	320	
Mini	40			40	80						
Bataviae	40			40	40						
Pomona				40							
Cynopteri							40				40
Patoc	40	40	40	640	80		40	640	320		5120

Results of comparison of M.A.T., Patoc and Difco Tests on Bovine sera.

Bovine Serum	Patoc Neat	(SAT) 1:10	Result	DIFCO 1	2	3	(SAT) 4	5	6	Result
1114	+4	+2	P	(±)	-	-	-	-	-	D
1118	+4	+1	P	(±)	-	-	-	-	-	D
1123	+3	tr	P	+	-	+	(±)	(±)	(±)	P
1129	+4	+4	P	±	-	-	(±)	(±)	-	D
1131	+3	tr	P	(±)	-	(±)	-	-	-	D
1135	+3	tr	P	-	-	-	-	-	-	N
1136	+3	tr	P	-	-	-	-	-	-	N
1138	+2	tr	D	(±)	-	-	-	-	-	D
1142	+1	-	D	+	-	-	-	-	-	P
1143	+4	+4	P	(±)	-	(±)	(±)	-	-	D
1148	+3	tr	P	(±)	-	-	-	-	-	D
1152	+3	tr	P	-	-	-	-	-	-	N
1154	+4	+4	P	(±)	-	+	-	(±)	(±)	P
1155	+3	-	D	-	-	-	-	-	-	N
1156	+2	tr	D	+	-	-	-	-	-	P
1157	+2	tr	D	(±)	(±)	(±)	(±)	(±)	(±)	D
1158	+2	tr	D	(±)	-	-	-	-	-	D
1160	+2	tr	D	+	(±)	(±)	(±)	(±)	(±)	P
1161	+2	tr	D	(±)	(±)	+	(±)	(±)	(±)	P
1164	+2	-	D	(±)	-	(±)	-	-	-	D
1168	+3	+2	P	(±)	-	-	-	-	-	D
1169	+4	+1	P	+	(±)	+	+	(±)	+	P
1173	+3	+1	P	+	(±)	+	+	+	+	P
1176	+3	tr	P	+	+	+	+	+	+	P
1177	+3	+1	P	(±)	-	(±)	-	-	(±)	D
1178	+4	+3	P	+	+	+	+	+	+	P
1180	+2	-	D	(±)	-	-	(±)	-	-	D

1181	+3	tr	P	+	-	+	+	+	+	P
1182	+3	+1	P	+	+	+	+	nd	+	P
1183	+2	-	D	-	-	-	-	nd	(±)	D
1184	+4	-	D	(±)	(±)	+	(±)	nd	+	P
1185	+4	+4	P	+	+	+	+	nd	+	P

N = Negative, D = Doubtful, P = Positive, nd = not done.

+1 = Particulate agglutination, +2 = Small aggregates and turbidity, +3 = Large aggregates and slight turbidity, +4 = Large aggregates and no turbidity, + = Positive, (±) = Doubtful, - = Negative.

M.A.T. Results on Bovine serum

(Titre expressed as reciprocal)

Serum No.	*Ict.*	*Sej.*	*Har.*	*Sax.*	*Cynop.*	*Grip.*	*Heb.*	*Mini*	*Patoc*
1123		200							
1131		200	200						
1138		200	200	200					
1142		200	200	200					
1152				100					
1154	100								
1158					200				100
1164			100	200					
1168						400			
1169		200	200						
1177		200	200	200					
1178		800	400	800			200	400	
1180		100	200	200					
1181		100	100				100		
1183				100					
1184		100							
1185		200	200	400					200

APPENDIX: X

SYNONYMS OF LEPTOSPIROSIS

Weil's disease	Seven day fever
Cane cutter's disease	Japanese autumnal fever
Rice field worker's disease	Fort Bragg fever
Hay maker's disease	Pretebial fever
Pea picker's disease	Mouse fever
Swineherd's disease	Canicola fever
Fish handler's disease	Marsh fever
Stuttgart disease	Swamp fever
Enzootic jaundice	Mud fever
Haemorrhagic jaundice	Field fever
"Yellows"	Water fever
Red water	Harvest fever

ACKNOWLEDGEMENTS

I would like to thank the many friends and colleagues who helped me during this work.

Firstly, I should like to thank Dr. Sheena Waitkins, the Director, PHLS Leptospira Reference Unit; WHO/FAO Collaborating Centre, England for her encouragement and for editing this work. I also wish to thank my supervisor, Dr. John Grant, for his encouragement and for sub-editing this work.

Many thanks also to all members of staff at the Leptospira Reference Unit; Mr. P. Friedman, Mr. M. Palmer, Mrs. W. Zochowski and Dr. J. Hookey for their technical assistance. Particular thanks to Mr. G. Taylor who took the photographs and produced the video film of my work. I also wish to thank the members of staff from the Histology Department, County Hospital, Hereford for their help.

Thanks to the Ministry of Agriculture, Fisheries and Food, and the Coypu Research Laboratory officials in Norwich and the trappers who enabled me to carry out the project.

I wish to thank the British Council and the Kenya Agricultural Research Institute; Veterinary Research Department without whose financial and moral support this project would not have been possible.

A very special word of thanks to Mrs. J. Cawley for her patient and efficient typing of the manuscript.

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