HUMORAL ECOLOGY OF LEPTOSPIROSIS IN DOGS

by

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LEPTOSPIROSIS

INTRODUCTION

Leptospirosis is an infectious disease of man and animals caused by the members of the genus Leptospira. The disease is universal in distribution (Chang et al., 1948; Martin, 1958; Galton et al., 1958b). It occurs in acute, subacute and chronic forms (Bloom, 1953; Mosier, 1957). A wide variety of animals including man are susceptible (Babudieri, 1958; Michna and Campbell, 1970) and infection is transmissible from animals to man. Leptospirosis is considered to be the world's most widespread contemporary zoonosis (Hoeden, 1964). Convalescent and chronic cases act as carriers by shedding the leptospires in urine for a considerable length of time (Stoenner, 1957a; Hoeden, 1958). Clinical diagnosis of leptospirosis is unreliable due to the variability of observed symptoms (Turner, 1967). Several diagnostic methods are available including darkfield examination, cultural and serological techniques, and animal inoculation. These methods are all time consuming (Larson, 1953; Stoenner, 1957a) and may be unreliable under certain conditions (Stiles and Sawyer, 1942; Bloom, 1953; Gochenour, 1953; Alexander et al., 1957).

Effective control of leptospirosis lies in detection of the carrier shedder. The efficiency and reliability of any method employed for diagnosis of leptospirosis is highly desirable. This has been accomplished to a limited extent by the development of fluorescent antibody techniques (FAT) (Anon., 1967). Sulzer found FAT to be superior to cultural or serological tests in detecting positive cases of leptospirosis (Sulzer et al., 1968).

Although a wide variety of animals are susceptible to leptospirosis, the major potential source of infection for man is dogs, cattle, swine, rodents and contaminated sewage water (Galton et al., 1962).

The objectives of this study were to:

(1) Randomly survey urine specimens from dogs brought to the Dykstra Veterinary Clinic, Kansas State University, for leptospires shedders by fluorescent antibody and culture techniques.

(2) Determine the minimum number of leptospires in dogs' urine which can be detected by fluorescent antibody techniques, and be isolated by culture.

(3) Satisfactorily control non-specific staining of urine solids.

PAPER 1:   DETECTION OF LEPTOSPIRES SHEDDER DOGS
           BY FLUORESCENT ANTIBODY TECHNIQUES

SUMMARY

Urine samples from 591 dogs brought to Dykstra Veterinary Hospital, Kansas State University, were surveyed for detection of leptospires shedders by culture and fluorescent antibody techniques (FAT). Leptospires were detected in urine of 19 dogs (3.1%) by FAT on Millipore filter membrane impression smears but not in centrifuged formalinized urine sediments nor by culture. In experimental infection of 4 dogs with L. canicola (Strain Moulton Dog Clone 36HP) leptospires were recovered from the blood of all 4 dogs, not only during the febrile stage, but also in one case when body temperatures were within normal ranges. All 4 experimentally infected dogs became shedders 9-16 days post inoculation. Leptospires were isolated from urine of 3 dogs in culture media and were demonstrated in all 4 dogs by FAT.

INTRODUCTION

Leptospirosis was first described by Weil in 1886[37] and the causative agent Leptospira icterohemorrhagiae was isolated by Ianda et al. in 1916.[18] Many species of domestic and wild carrier animals act as a source of infection to man and other susceptible animals.[16] Carrier shedder dogs, cattle, pigs[13] and rats in slums[40] are common. Detection of the carrier shedder is an important step in the effective control of leptospirosis.[39] Various diagnostic methods such as darkfield examination of body fluids and tissue smears, cultural, animal inoculation, serological and fluorescent antibody techniques have been described.[1, 3, 5, 8-10, 13, 14, 34-36, 38, 39] Darkfield examination of urine is not recommended as a single diagnostic test.[3, 13] Culture of

blood is limited to the first week of illness[3, 13] and as such is not suitable

for the detection of carriers. Culture techniques for voided urine[24] and

bladder tapping[23] have been successfully employed but frequent contamination

has been a limiting factor. Serological methods have been described but none

are satisfactory for detecting an active shedder. White et al.[39] reported

isolation of leptospires from 15 of 46 serologically positive dogs. Demon-

stration and isolation of leptospires from urine are definitive methods for

detection of carrier shedders. Fluorescent antibody techniques (FAT) have

been applied to fresh[38] and formalinized urine samples stored at room temper-

ature for up to 9 months[9] and in tissues preserved in formalin up to 618

days.[10] Several surveys on the incidence of leptospirosis in the canine

population of the U.S.A. have been reported, the average incidence has been

12%[33] (range from zero[26] to 38%[30]). The object of this study was to randomly

survey urine samples of dogs brought to Dykstra Veterinary Hospital, Kansas

State University, for leptospires shedders by cultural and FAT.

REVIEW OF LITERATURE

Jungherr[20] reported the first case of leptospirosis in dogs in the

United States. Meyer et al.[25] isolated _Leptospira canicola_ and Randall and

Cooper[29] isolated _Leptospira icterohemorrhagiae_ from dogs initially in the

United States. Meyer et al.[25] reported the incidence of leptospirosis to be

25% and Greene[15] 29% in dogs surveyed in Northern and Southern California.

Raven[30] reported an incidence of 38% in rural and 28% in urban dog populations

of Philadelphia. Jones et al.[19] reported an overall incidence of 1.33% in

4,368 dogs surveyed from several different states. Newman[28] found 29.8% posi-

tive in 500 dogs surveyed in the vicinity of Lansing, Michigan. Bohl and

Ferguson[6] reported 31.6% positive in 79 dogs surveyed in Ohio. Byrne[7] stated

that the incidence of leptospirosis in dogs in the U.S.A. ranges from 3-38% with 90% of them being positive for L. canicola and 10% for L. icterohemorrhagiae. Alexander et al.[2] reported a survey on 1,017 dogs obtained from different states out of which 838 were normal German Shepherd dogs; 11% were positive by serological techniques. He observed that a higher percentage of the dogs positive for L. icterohemorrhagiae came from east, north and central states. Mosier[26] stated that incidence of L. canicola varied from zero to 26% and L. icterohemorrhagiae from zero to 8%. Shill[31] reported that 26.4% of stray dogs and 13.3% of purebred dogs of 226 dogs surveyed from San Joaquin and Contra Counties in California were serologically positive. Dolowy and Reich[11] noted that, of 659 dogs surveyed, 37.3% from Chicago and 32.2% from other states were positive. Kravis and Ivler[22] found 41.4% positive in 79 dogs surveyed from Syracuse, New York. White et al.[39] reported 23.8% serologically positive in 193 stray dogs surveyed from a Florida metropolitan area. They isolated leptospires from urine specimens of 17 (11%) of 156 examined and from 15 of 46 serologically positive dogs. Hubbert and Shotts[17] reported isolation of L. canicola from urine of 10 of 19 healthy dogs in a kennel. Thomas et al.[33] surveyed 1,161 German Shepherd dogs obtained from 46 states. The average incidence was found to be 12.4% (range 6.6 to 22.2%). Fenberg et al.[12] found 10% of 240 mongrel dogs and 7% of 306 normal Beagle dogs to be positive by serological techniques.

MATERIALS AND METHODS

Antileptospiral serum was prepared in rabbits in accordance with the procedures employed by the Walter Reed Army Institute of Research, Washington, D. C.[1] Successive doses of 1 ml, 2 ml, 4 ml and 6 ml of 5-day-old

culture of L. canicola[a] (Strain Moulton Dog Clone 36HP) grown in Stuart's medium[b] were inoculated intravenously at weekly intervals into normal adult rabbits. On day 6 following the last inoculation the rabbits were exsanguinated and serum harvested. Pooled antibody titers were determined by plate agglutination test and microscopic agglutination test.[1, 13] The pooled titer was found to be 1:320 complete, and 1:640 incomplete by plate agglutination test, and 1:12,500 by the microscopic agglutination test. Labeling of the antibodies with fluorescein isothiocyanate, preparation of acetone dried dog tissue powder and adsorption of the conjugate with tissue powder were performed by the method described by Kawamura.[21] The optimum staining titers of adsorbed and nonadsorbed conjugates were determined by diluting with .01M phosphate buffered saline (PBS) pH 7.2, using four fold dilutions and staining positive control slides with each dilution. The average optimum titer was 1:16 based on maximum specific and minimum nonspecific fluorescence.

Collection and Processing of Urine Samples

Midstream voided urine was collected from 591 dogs, including strays, brought to Dykstra Veterinary Hospital, Kansas State University. One drop of urine was immediately inoculated directly into 5 ml Stuart's medium.[24] The pH of urine was adjusted to 7.2 - 7.6[3] by using 2N NaOH and buffered[38] with 1 ml of PBS .01M pH 7.6 for every 10 ml of urine. In the laboratory 15 ml of urine was centrifuged at 381 g (1,500 RPM) in Sorvall G-L-C-1 centrifuge[c] for

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[a]Obtained by the courtesy of Dr. Alexander, Chief WHO/FAO Leptospirosis Reference Laboratory, W.R.A.R.I., Washington, D. C.

[b]Difco Laboratories, Detroit, Michigan.

[c]Sorvall, New Town, Connecticut.

15 minutes. Approximately 2 ml of supernatant were filtered through a 13 mm, 0.45 u pore size, Millipore filter membrane in a Swinny holder.[d] Four to six drops of filtrate were inoculated into Stuart's media after discarding the first 2-3 drops. The culture tubes were incubated at 30°C and examined under darkfield after one week. If the tubes were negative, they were examined again at the end of the 4th week before considered negative. The remaining portion of the supernatant was formalinized with 10% buffered neutral formalin (BNF) to 0.8% final concentration.

Smears were made from the filter membranes and the sediment from centrifuged formalinized urine on slides previously treated with 1% gelatin. The sediment of the formalinized samples was collected for smears after centrifuging at 1,522 g (3,000 RPM) in Sorvall G-L-C-1 centrifuge for 45 minutes. Both types of smears were stained by direct FAT,[27] counterstained with Lissamine Rhodamine FA[e] and immediately examined. If an animal proved to be a suspected carrier by FAT, an attempt was made to obtain a serum sample for serological titer determinations.

Experimental Studies

Four dogs of mixed breed, 6 to 8 months old, free from leptospirosis (by FAT on urine) and leptospiral serum antibodies (by plate agglutination test) were inoculated intraperitoneally with 4 daily doses of 4.5 ml of 5-7-day-old culture of L. canicola (Strain Moulton Dog Clone 36HP) grown in Stuart's medium containing 1.6×10^8 organisms per ml. Daily morning and evening rectal temperatures were recorded and blood specimens cultured in

[d]Millipore Corporation, Bedford, Massachusetts.

[e]Difco Laboratories, Detroit, Michigan.

Stuart's and Fletcher's[f] media during the febrile stage. After the 8th day voided and catheterized urine specimens were collected, and one drop was directly inoculated into Stuart's medium. The urine was serially diluted in .01M PBS pH 7.2 from 10^{-3} to 10^{-6} and a drop from each dilution was inoculated into 5 ml of Stuart's medium.[23, 24] The urine was first examined under darkfield and processed as described.

RESULTS

Urine samples from 591 dogs were examined by cultural and FA techniques for leptospires shedders between November 1970 and August 1971. The findings are summarized in Table 1. Plate agglutination tests for leptospiral antibodies on serum samples from 6 suspected shedder dogs were negative.

The results of blood and urine examinations on experimentally infected dogs are summarized in Tables 2 and 3. Leptospires recovered from the experimental dogs were reported by the World Health Reference Laboratory, Veterinary Division, Walter Reed Army Institute of Research, Washington, D. C., as belonging to serotype canicola (Strain Moulton Dog Clone 36 HP).

DISCUSSION

Most surveys reported are based entirely on serological studies. Positive serological results do not indicate the carrier shedder status of the dog. White et al.[39] reported isolation of leptospires from urine of only 32% of serologically-positive dogs. Demonstration and isolation of leptospires in urine are the definitive methods of choice for the detection of a shedder. Limiting factors in isolation of leptospires from urine in culture media

[f]Difco Laboratories, Detroit, Michigan.

include frequent contamination, too few leptospires in urine,[13] acid pH,[13] intermittent shedding,[3] presence of leptospiral antibodies in urine,[32] time delay in processing specimens,[39] and a very short period of survival of leptospires in urine outside the host.[16] In absence of isolation, leptospires may still be successfully demonstrated by FAT. In this survey 3.1% of the dogs surveyed were found to be positive by FAT on Millipore filter membrane impressions, but smears from centrifuged formalinized sample sediments and culture techniques were negative. A possible explanation would be the presence of too few leptospires in urine. Millipore filter membrane filtration offers a high concentration of leptospires in a small area. If the number of viable and intact leptospires were sufficiently high, cultural techniques and formalinized sediments should have given positive results. This hypothesis is supported by limited but successful isolation by culture technique and demonstration of leptospires in formalinized samples of urine by FAT from experimentally infected dogs. In control specimens leptospires could be demonstrated by FAT in formalinized urine samples stored for 9 months at room temperature without the loss of fluorescence. Other limiting factors in this survey were non-availability of multiple samples and institution of antibiotic treatment. It has been reported,[3] and also observed in this study, that dogs may be intermittent shedders. Antibiotics have been reported to be effective, though temporarily, in eliminating the shedder state.[4, 17, 36] It may be observed from Table 2 that leptospires were recovered from blood of all 4 dogs not only during the febrile stage, but also in one case when the temperature was within the normal range (Dog #96). The dogs became shedders 9 to 16 days post-inoculation. Dogs #96 and #99 gave positive results by culture earlier than by FAT or darkfield examination. Dogs #97 and #98 gave positive

results by FAT earlier than by culture or darkfield examination. White et al.[39] have reported similar findings.

This study supports the findings of previous workers that FAT is a rapid and relatively accurate method for demonstrating leptospires in canine urine. Leptospires can be isolated experimentally in pure culture by filtration through Millipore filter membranes and by simple dilution techniques. Leptospires may be demonstrated in positive formalinized urine samples for at least 9 months kept at room temperature. The FAT is genus specific and does not provide positive identification as to serotype and strain of leptospires. In conducting a survey for the detection of carrier shedder, FAT should be applied first on urine samples and if found positive further attempts should be made to isolate leptospires by bladder tapping or dilution techniques.

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Table 1. Results of Survey of Canine Urine Samples for Leptospires Shedders
by Fluorescent Antibody and Cultural Techniques.

Total number examined	FAT on Millipore filter membrane impression smears	FAT on formalinized sample smears	Urine culture[*]
591	19 pos.	neg.	neg.

[*]Stuart's medium.

Pos. = Positive for leptospires.

Neg. = Negative for leptospires.

Table 2. Results of Blood Examination by Darkfield and Culture on Dogs Experimentally Infected With *Leptospira canicola* (Strain Moulton Dog Clone 36HP).

P.I. Day	96 Body temp. C°	Dark-field	Culture*	97 Body temp. C°	Dark-field	Culture*	98 Body temp. C°	Dark-field	Culture*	99 Body temp. C°	Dark-field	Culture*
2	NE	NE	NE	A.M. 39.4	Neg.	Pos.	NE	NE	NE	NE	NE	NE
				P.M. 41.6	Pos.	Pos.						
3	39.3	Neg.	Pos.	40.0	Neg.	Pos.	41.0	Neg.	Pos.	39.8	Pos.	Pos.
4	39.8	Neg.	Pos.	38.6	NE	NE	39.2	Neg.	Pos.	39.3	Neg.	Pos.
5	39.2	NE	Pos.				39.0	NE	NE	39.6	NE	Pos.
6	38.6	NE	Pos.							39.5	NE	Pos.
7	38.6	NE	NE							38.4	NE	NE

*Stuart's and Fletcher's media.

P.I. Day = Post inoculation day.   NE = Not examined.   Neg. = Negative for leptospires.

Pos. = Positive for leptospires.

Table 3. Results of Urine Examination by Darkfield, Fluorescent Antibody Techniques (FAT) and Culture on Dogs Experimentally Infected with _Leptospira_ _canicola_ (Strain Moulton Dog Clone 36HP).

Dog #	96			97			98			99		
Post inoculation day	Dark-field	FAT	Culture*	Dark-field	FAT	Culture*	Dark-field	FAT	Culture*	Dark-field	FAT	Culture*
9	NE	NE	NE	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.
13	Neg.	Neg.	Pos.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	Pos.
14	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.
15	Neg.	Neg.	Neg.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	Neg.
16	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	NE
17	Neg.	Neg.	Neg.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	NE
18	NE	NE	NE	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	NE	NE	NE
19	NE	NE	NE	NE	NE	NE	Neg.	Neg.	NE	NE	NE	NE
20	Neg.	Neg.	Neg.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	Neg.
21	Neg.	Neg.	Neg.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	NE
22	Neg.	Neg.	Neg.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	NE
23	Neg.	Neg.	Neg.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	NE
24	NE	NE	NE	NE	NE	NE	Neg.	Neg.	Neg.	NE	NE	NE
27	Neg.	Pos.	Neg.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	NE

Table 3 (Continued)

Dog #	96			97			98			99		
Post inocula-tion day	Dark-field	FAT	Culture*	Dark-field	FAT	Culture*	Dark-field	FAT	Culture*	Dark-field	FAT	Culture*
28	NE	NE	NE	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	NE	NE	NE
29	Neg.	Pos.	Neg.	Pos.	Pos.	Neg.	NE	NE	NE	Pos.	Pos.	Pos.
30	NE	NE	NE	Pos.	Pos.	Pos.	Neg.	Neg.	Neg.	NE	NE	NE
31	Neg.	Neg.	Neg.							Pos.	Pos.	Neg.
37	Pos.	Pos.	Pos.							Pos.	Pos.	Pos.
41	Neg.	Neg.	Pos.							Neg.	Neg.	Neg.
43	Pos.	Pos.	Pos.							Neg.	Neg.	Pos.

*Stuart's medium.

NE = Not examined.   Neg. = Negative for leptospires.   Pos. = Positive for leptospires.

PAPER 2:   MINIMUM NUMBER OF LEPTOSPIRES IN URINE REQUIRED
           FOR DETECTION

SUMMARY AND CONCLUSIONS

Experiments indicated that as few as 10 viable leptospires in canine urine can be successfully recovered by culture. These findings further indicated that if fresh urine is available, isolation of leptospires should always be attempted by filtering the urine through Millipore filter membranes or by simple dilution techniques. A smear can be readily made from the filter membrane and subjected to fluorescent antibody techniques (FAT). If older urine specimens or chemically preserved urine specimens are received, FAT is the technique of choice.

For both darkfield and FAT the possibility of chance distribution of organisms in the specimen makes the use of multiple samples preferable when the number of organisms per ml is 2.5×10^3 or less. Darkfield examination of non-viable and fragmented leptospires is subject to potential misdiagnosis and is not recommended.

INTRODUCTION

Leptospirosis is one of the better known zoonoses. Many species of wild and domestic leptospires shedder animals act as a source of infection to susceptible animals and man.[3] The dog is reported to be the principal source of canicola fever infection in man.[12, 15] It may remain as a shedder for up to 4½ years.[17] Bryne[7] stated that the incidence of leptospirosis in dogs in the United States ranged from 3-38%, and that 90% of those positive have Leptospira canicola and 10% have Leptospira icterohemorrhagiae. Meyer et al.[20] reported leptospirosis in two veterinarians and isolated L. canicola from urine of one. Haunz and Cardy[14] reported 9 cases of canicola fever in

one family. Their pet dog was a carrier shedder. Cockburn et al.[8] and Williams et al.[31] reported an outbreak of leptospirosis in man due to swimming in contaminated water. Domestic animals in the area had serological titers against L. canicola. Bigler et al.[5] reported that most of the cases of human leptospirosis in Florida included a history of contact with dogs, rats, cattle or pigs. The urinary system is the route of excretion of leptospires.

Demonstration and/or isolation of leptospires from urine are the definitive methods of detection of carrier dogs.[13] Menges et al.[18, 19] reported successful isolation of leptospires from urine by both bladder tapping and dilution techniques.

Baker and Baker[4] reported that 9 or less virulent leptospires were often lethal to hamsters. There is no report available regarding the minimum number of leptospires present in urine necessary for successful isolation. The object of this study was to determine the minimum number of leptospires in urine necessary for isolation in culture media and to compare the culture results with both darkfield examination and fluorescent antibody techniques (FAT).

MATERIALS AND METHODS

Leptospira canicola[a] (Strain Moulton Dog Clone 36HP) was used in this study. Cultures for routine work were maintained in Stuart's medium[b] and stock cultures were maintained in Fletcher's semisolid medium.[b] The cultures maintained in Stuart's medium were transferred at 5-7 day intervals and were

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[a]Obtained from WHO/FAO Leptospirosis Reference Laboratory. W.R.A.R.I., Washington, D. C.

[b]Difco Laboratories, Detroit, Michigan.

used in this study. The leptospires counts were made with a Petroff-Hausser bacteria counter.[c] Fresh voided urine from dogs free from leptospirosis (by FAT on urine) and leptospiral antibodies (by plate test on serum) was collected and the pH adjusted to 7.2 - 7.6 by using 2N NaOH or dilute hydrochloric acid. Ten ml of the specimens were then centrifuged at 381 g (1,500 RPM) for 15 minutes in Sorvall G-L-C-1 centrifuge.[d] Three ml of the supernatant was then filtered through a Millipore filter[e] 13 mm .45 u pore size in a Swinny holder to remove contaminants.[23] Serial dilutions of a 5-7-day-old culture were made in Stuart's medium and the final dilutions in filtered urine. Known numbers of leptospires in urine, starting with 1.5×10^7, were inoculated into each of the 3 test tubes containing 5 ml Stuart's medium and incubated at 30°C. They were examined under darkfield at weekly intervals for 4 weeks before they were considered negative. All trials were duplicated twice. Comparative studies were made with darkfield microscopy and FAT. A drop of urine from each well-mixed dilution of leptospires was first examined under darkfield with 100X and 400X magnification. The darkfield examination was considered positive only when actively motile leptospires were seen. If the first examination was negative 3 more drops were examined before the specimen was considered negative. Five ml of each dilution of urine was centrifuged at 1,522 g (3,000 RPM) for 45 minutes in Sorvall G-L-C-1 centrifuge. Two smears were made from the sediment of each dilution on slides pretreated with 1% gelatin. They were then stained by direct FAT [22] and examined.

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[c]Arthur H. Thomas Co., Philadelphia, Pennsylvania.

[d]Sorvall, New Town, Connecticut.

[e]Millipore Corporation, Bedford, Massachusetts.

RESULTS

The findings are summarized in Tables 1 and 2.

DISCUSSION

As few as 10 viable organisms were enough for successful isolation in culture media provided the urine was free from significant antibodies,[26] contaminants[28] and the pH adjusted between 7.2 to 7.6. Darkfield examination and FAT were both positive on urine samples containing 2,500 leptospires per ml. Both methods failed to detect the leptospires in higher dilutions.

Each method has certain limitations. Urine for darkfield examination must be fresh and examined immediately to detect actively motile leptospires. Other limitations are too few leptospires in urine and presence of pseudo-spirochetes.[1] These factors result in misdiagnosis or failure to diagnose an otherwise positive case.[12] Darkfield microscopy is not recommended as a single diagnostic test for leptospirosis.[1, 12] Successful isolation of leptospires from voided urine and bladder tapping have been reported.[18, 19] Menges et al.[19] reported successful isolation by diluting urine in phosphate buffered saline (PBS) up to 10^{-6}. White et al.[30] reported that when too few leptospires were present, bladder tapping was most successful. Both techniques have been successfully applied by other workers.[11, 16, 24, 25, 27, 30] This study revealed that as few as 10 organisms were sufficient for cultural isolation under the conditions of this experiment. Carrier animals may shed in urine as many as 10^8 leptospires per ml.[2] If an animal is shedding as few as 1,000 viable leptospires per ml, 4-6 drops of filtrate of fresh urine filtered through a Millipore filter membrane should provide enough numbers of organisms for successful isolation.

Application of FAT for the demonstration of leptospires in urine has been previously reported.[6, 9, 21, 29] The advantages of FAT are twofold.[28] Leptospires are stained so that they may be seen under fluorescent microscopy and the technique can be applied to materials which are unsuitable for culture.[10] Nonviable and viable organisms both fluoresce specifically. White et al.[30] recommended FAT for detecting a shedder but preferred culture of urine collected by bladder tapping when few leptospires were present. Boulanger and Robertson[6] reported that FAT was less effective with fewer leptospires in urine, lower in efficiency than culture and nearly as effective as darkfield examination. In this study (Table 2) darkfield examination was as effective as FAT on fresh specimens but both failed to detect leptospires with fewer numbers when culture technique was successful (Table 1). A possible explanation for the failure of darkfield examination and FAT when fewer leptospires are present could be chance distribution. Leptospires may not be present in a particular drop when examined under darkfield or by FAT. Moulton and Howarth[21] reported similar findings.

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Table 1. Results of Isolation of <u>Leptospira canicola</u> (Strain Moulton Dog
Clone 36 HP) from Canine Urine in Stuart's Medium.

Number of leptospires inoculated	Number of tubes inoculated	Number of tubes positive*	Number of tubes negative**	Number of tubes contaminated
1.5×10^7	12	11	0	1
1.2×10^7	12	10	1	1
9×10^6	12	11	0	1
6×10^6	21	13	5	3
4×10^6	21	17	0	4
2×10^6	21	17	0	4
1.5×10^6	6	6	0	0
10^5	6	6	0	0
5×10^4	6	6	0	0
4×10^4	9	6	0	3
2×10^4	9	7	0	2
10^4	9	7	0	2
8×10^3	9	8	0	1
4×10^3	9	7	0	2
2×10^3	9	6	0	3
1.5×10^3	9	6	0	3
10^3	9	9	0	0
5×10^2	9	8	0	1
4×10^2	9	6	2	1
2×10^2	9	6	2	1
10^2	9	6	3	0
40	11	7	4	0
20	11	4	7	0
10	11	7	4	0

*Growth of leptospires in culture medium.

**No growth of leptospires in culture medium.

Table 2. Results of Darkfield Microscopy and Fluorescent Antibody Techniques
(FAT) on Canine Urine Containing <u>Leptospira</u> <u>canicola</u> (Strain Moulton
Dog Clone 36 HP).

Undiluted leptospires count	Dilution	Number of leptospires per ml.	Darkfield examination	FAT
1.8×10^8/ml	$1:18 \times 10^3$	10^4	Pos.*	Pos.
	$1:36 \times 10^3$	5×10^3	Pos.	Neg.**
	$1:72 \times 10^3$	2.5×10^3	Pos.	Pos.
	$1:18 \times 10^4$	10^3	Neg.	Neg.
	$1:36 \times 10^4$	5×10^2	Neg.	Neg.
	$1:72 \times 10^4$	2.5×10^2	Neg.	Neg.
	$1:18 \times 10^5$	10^2	Neg.	Neg.
	$1:18 \times 10^6$	10^1	Neg.	Neg.
	$1:36 \times 10^6$	5	Neg.	Neg.

*Pos. = Positive for presence of leptospires.

**Neg. = Negative for presence of leptospires.

PAPER 3: NONSPECIFIC FLUORESCENCE IN CANINE URINE
          EXAMINED FOR LEPTOSPIRES

SUMMARY

During a survey of individual urine samples from 384 male dogs for possible leptospires shedders, nonspecific staining by spermatozoa was found to be a problem and, at times, misleading when stained with unadsorbed fluorescein isothiocyanate (FITC) conjugates. Nonspecific staining was eliminated by adsorbing the FITC conjugate with acetone dried dog testis tissue powder. Adsorption with mouse and dog liver acetone dried powders however was less effective.

INTRODUCTION

In a random survey of canine urine samples to detect leptospires shedders, spermatozoa and fragments were found to be a constant source of nonspecific fluorescence. Tail fragments of disintegrated spermatozoa may be confused with leptospires and clusters of intact spermatozoa may mask the presence of any leptospires in the vicinity. Nonspecific fluorescence in fluorescent antibody techniques (FAT) has previously been reported to be a limiting factor in routine application.[8] It may be caused by unreacted fluorescent materials, conjugated serum proteins, unwanted conjugated antibodies, improper fixation of tissue or allowing the specimen to dry during staining.[7, 9, 13] This paper reports the use of acetone dried testis powder to reduce the nonspecific staining of urine sediments.

REVIEW OF LITERATURE

Coons and Kaplan[5] adsorbed conjugates with normal tissue powder to reduce nonspecific staining. They reported that the species from which the

tissue originated was not significant but that from homologous species was preferable. Sheldon[14] used bone marrow tissue powder in preference to liver tissue powder to eliminate the nonspecific staining of neutrophils. Moulton and Howarth[12] reported success with normal hamster kidney and liver tissue powder. Dacres[6] diluted conjugated antibody beyond the point of nonspecific fluorescence. Coffin and Maestrone[4] used acetone dried canine liver powder for work with canine specimens. Maestrone[10] used acetone dried powders of fresh and formalin fixed tissues from dog, cat, rabbit and whole chick embryo. Chernukha and Korn[3] reported adsorption of conjugates with killed heterologous serotypes of leptospires eliminated nonspecific staining of heterologous serotypes.

MATERIALS AND METHODS

Antileptospiral serum was prepared in rabbits in accordance with the procedures employed by the Walter Reed Army Institute of Research.[1] Intravenous inoculations were made in normal adult rabbits with a live 5 day old culture of Leptospira canicola[a] (Strain Moulton Dog Clone 36HP) grown in Stuart's medium.[b] Serum antibody titers were determined by plate agglutination and microscopic agglutination tests. The serum antibodies were labeled with fluorescein isothiocyanate (FITC) by the method described by Kawamura.[9]

Adsorption of the Conjugate with Acetone Dried Tissue Powders

Fresh testis and liver tissues from dogs free from leptospiruria (by FAT on urine) and leptospiral serum antibodies (plate agglutination test) were

[a]Received by the courtesy of Dr. A. D. Alexander, Chief WHO/FAO Leptospirosis Reference Laboratory, W.R.A.I.R., Washington, D. C.

[b]Difco Laboratories, Detroit, Michigan.

collected after exsanguination. Adsorption of leptospira conjugates with acetone dried testis and liver tissue powders was carried out by accepted techniques.[7, 9, 13]

Collection and Processing of Urine Specimens

Midstream urine was collected from dogs brought to the Dykstra Veterinary Hospital, Kansas State University, for observation and a treatment as required. A drop of undiluted urine was immediately inoculated into 5 ml of Stuart's medium.[11] The pH of urine was adjusted to 7.2 to 7.6 by using 2N NaOH or dilute HCl.[2] It was then buffered with .01M phosphate buffered saline (PBS) pH 7.6. One ml of buffer was added to every 10 ml of urine.[15] It was then centrifuged at 381 g (1,500 RPM) in Sorvall G-L-C-1 centrifuge[c] for 15 minutes. Two ml of the supernatant were filtered through a Millipore filter[d] 13 mm .45 u pore size in a Swinny holder.[15] Six to eight drops of filtrate were inoculated into Stuart's medium and the remaining portion of the supernatant was formalinized with 10% buffered neutral formalin (BNF) to .8% final concentration. Smears were made on slides, previously treated with 1% gelatin, from filter membranes and the sediment of the formalinized portion after centrifuging at 1,522 g (3,000 RPM) in Sorvall G-L-C-1 centrifuge for 45 minutes and stained by the direct FAT.[13] Positive control smears containing leptospires and spermatozoa were stained with FITC conjugates previously adsorbed with acetone dried mouse liver, dog liver and dog testis tissue powder.

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[c]Sorvall, New Town, Connecticut.

[d]Millipore Corporation, Bedford, Massachusetts.

RESULTS

Results are summarized in Table 1. Sixteen positive control smears were stained each with acetone dried mouse liver, dog liver, and dog testis tissue powder adsorbed conjugates. Two hundred and twelve urine specimens from male dogs were stained with unadsorbed conjugate and 172 with conjugate previously adsorbed with acetone dried dog testis tissue powder. In all smears stained with unadsorbed FITC conjugate, spermatozoa had nonspecific yellow-green fluorescence approaching, but not identical to, the specific apple-green fluorescence of FITC stained leptospires. In smears stained with testis tissue powder adsorbed FITC conjugate, nonspecific fluorescence by spermatozoa was not observed. Occasionally the heads of spermatozoa were faintly fluorescent. It may be observed from Table 1 that acetone dried mouse and dog liver tissue powders both were ineffective in eliminating nonspecific fluorescence due to spermatozoa.

DISCUSSION

Of the several methods described for elimination of nonspecific fluorescence, adsorption of FITC conjugates with acetone dried liver tissue powder is most commonly used.[9] It has been reported to be more efficient than diethylaminoethyl (DEAE) cellulose fractionation in removing unwanted fluorescent conjugates.[13] Though the origin of tissue was not considered to have significant effect in eliminating the nonspecific fluorescence,[5] tissues from homologous species were preferred.[10] In this study mouse and dog acetone dried liver powders were not as effective as dog acetone dried testis tissue powder. This study also indicates that tissue from homologous species and homologous organs and tissues are preferable especially for the detection of leptospires in canine urine.

Nonspecific staining of spermatozoa tail fragments may lead to misdiagnosis or failure to diagnose an otherwise positive case of leptospirosis. This problem was eliminated with adsorption of the FITC conjugate with acetone dried testis tissue powder.

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Table 1. Results of Positive Control Urine Solids Smears Containing Leptospires and Spermatozoa Stained by Fluorescent Antibody Techniques with Conjugates Previously Adsorbed with Acetone Dried Tissue Powders.

Dilution of the adsorbed conjugate	Number of slides stained with each tissue powder adsorbed conjugate	Mouse liver powders	Dog liver powders	Dog testis powders
		Results	Results	Results
1:4	4	Spermatozoa stained bright yellowish-green.	Spermatozoa stained bright yellowish-green.	Heads of spermatozoa stained faint yellowish-green.
1:8	4	Similar findings.	Similar findings.	Similar findings.
1:16	4	Similar findings.	Similar findings.	Similar findings.
1:36	4	Similar findings.	Similar findings.	Similar findings.

ACKNOWLEDGMENTS

VITA

Pralhadrao Narasingrao Kamalapur, the son of Narasingrao Shrinivasrao Kamalapur, was born on February 9, 1927, at Gadag, District Dharwar, Mysore State, India. He was educated at Dharwar in K.E.B.'s High School and Karnatak College. He graduated from the University of Bombay, India, with B. Sc. (Vet.) degree in 1951. From 1951 to 1960 he worked for the States of Bombay and Mysore as veterinary officer. In 1960 he came to Kansas State University, Manhattan, Kansas, and obtained an M. S. degree in surgery and medicine in 1961. On his return to India he joined the faculty of the Mysore Veterinary College at Bangalore as lecturer in preventive medicine and later joined the University of Agricultural Sciences at Bangalore as assistant veterinary scientist.

He and Nirmala were married in 1954. They have three daughters, Jayashree, Ratnaprabha and Shubha.

APPENDIX

REVIEW OF LITERATURE

Weil (1886) was the first to describe leptospirosis clinically which
was hitherto considered the same as infectious jaundice. He differentiated
leptospiral jaundice from other infectious jaundices on the basis of a study
of 4 cases in 1870 and 1872. In the 4 cases he studied, there were febrile
responses, enlarged livers and spleens, jaundice, and renal involvement. He
was not able to demonstrate the infective agent.

Landouzy (1883) had actually described the disease three years
earlier in men who worked in sewers. He attributed the disease to emanations
from sewage.

Goldschmit (1887) was the first to use the term "Weil's disease" for
the infectious jaundice condition described by Weil. Soon after recognition
of the disease by Weil it was reported by several other workers from differ-
ent parts of the world. Young (1889) reported a case of Weil's disease in a
patient returning from training in Southwest England, describing the symptoms
in detail. Jaeger (1892) reported the disease in 9 soldiers at Ulm, Germany.
He attributed the disease to bathing in a river. Chowdry (1903) recorded 588
cases in Andaman Islands between 1892 and 1903 which he considered to be
Weil's disease. He reported a mortality rate of 13 percent. He observed that
relapses were uncommon in recovered cases. He attributed the disease to a
sudden or prolonged exposure to rain or working in brick or rice fields.

In all the reports cited, the diagnoses were based on clinical
symptoms. Stimson (1907) described an organism which he named "interrogans"
in sections of a kidney from a patient who was believed to have died of yellow

fever in New Orleans. Later studies of his original description and photographs revealed that the organisms he observed and described were actually leptospires. The infective agent was not associated with infectious jaundice until 1914 when Inada et al. (1916) observed spirochetes in the liver tissue of a guinea pig inoculated with blood from a patient suffering from Weil's disease. They were successful in recovering the organisms from 13 out of 17 cases by animal inoculation. They could not recover spirochetes from patients suffering from certain other infections with jaundice. They concluded that the cause of Weil's disease was a spirochete and named it Spirocheta icterohemorrhagiae. Their experiments with guinea pig inoculations revealed positive results when the blood of the patient was inoculated into a guinea pig within the first 7 days after the onset of the disease and in no case after the 12th day. Hübener and Reiter (1915-1916) and Uhlenhuth and Fromme (1915-1916) discovered the agent independently in Germany. Hübener and Reiter were successful in producing the disease in guinea pigs by inoculating them with blood from patients suffering from Weil's disease. They were also successful in producing the disease in monkeys and rabbits, and in guinea pigs by inoculating the urine of the patients obtained on the 15th day of illness. They identified the organism by darkfield examination and named it "Spirocheta nodosa." Uhlenhuth and Fromme transmitted infection from man to guinea pigs. They were the first in Europe to demonstrate active spirochetes under darkfield from liver tissue. They named the organism "Spirocheta icterogenes." This early work was promptly confirmed by others. Leptospirosis was subsequently diagnosed by Stokes and Ryle (1916) and also by Dawson and Hume (1916) in British soldiers. Costa and Troisier (1916) reported the disease in French troops and Sisto (1917) reported it in Italian workers.

These workers were able to infect guinea pigs with inoculations of blood from patients.

Noguchi (1917) isolated Leptospira icterohemorrhagiae from rats in New York City. Since then numerous serotypes and strains of leptospira have been isolated from a wide range of domestic and wild animals, arthropod vectors, and birds. New species of reservoir hosts are still being added to the list (Babudieri, 1958; Galton et al., 1958a; Turner, 1967).

I Morphology of Leptospira

All serotypes and strains of leptospires have a similar morphology. Wolbach and Binger (1914) described the morphological characters of the L. biflexa, a nonpathogenic leptospirum, which had been isolated from stagnant water. They were 6 to 20 u in length and .03 to .2 u in diameter. Lengths up to 40 u were frequently observed. The spirals were fine and close with an amplitude of approximately 0.5 u. One or both ends were hooked. The organism has also been studied under electron microscopy. Electron microphotographs revealed that a leptospirum consists of an axial filament coiling spirally around a protoplasmic spiral and an enveloping sheath. Leptospires are motile by 3 types of movements: first, rotation around the long axis; second, to and fro; and third, snake-like or sinuous which is seen only in semisolid media (Alston and Broom, 1958; Merchant and Packer, 1967; Turner, 1970; Alexander et al., 1970).

A. Staining.

Spirochetes (including leptospires) can be demonstrated in tissue impressions and films from body fluids by Fontana's silver impregnation method and Giemsa stain. They can be observed in tissue sections by

Levaditi's silver impregnation method and Kerr's modification of Warthin
Starry method (Alexander et al., 1970; Sonnenwirth, 1970; Turner, 1970).

B. Filterability.

Leptospires are readily filterable through Berkfield V and N candles
and through membrane filters with pores of .22 mu, .30 mu, and .45 mu
(Rittenberg et al., 1958; White and Ristic, 1959; Turner, 1970).

C. Classification.

Noguchi (1917) proposed for creation of a new genus Leptospira on the
basis of specific features which differentiated them from other spirochetes.
They were (1) minute elementary sprials, (2) depth of the spiral not exceed-
ing the diameter of the body, (3) absence of a terminal flagella, and
(4) resistance to 10 percent saponin. The genus Leptospira is generally
divided into two main groups — pathogens and saprophytès. In 1963 the taxo-
nomic subcommittee on Leptospira recommended that they may be regarded as two
species. Accordingly the pathogenic leptospires were designated as L. inter-
rogans, and the saprophytic as L. biflexa. However, this classification is
not rigid and is not generally accepted as some apparently pathogenic lepto-
spires have characteristics similar to those of the nonpathogenic biflexa
strains. Different serotypes and strains within each of the two groups are
classified by means of cross-agglutination reactions and cross-agglutinin
absorption studies (Turner, 1967). Until 1967, 16 serogroups and 119 sero-
types were recognized (Turner, 1967) and by 1970 more than 130 serotypes of
parasitic and pathogenic strains were known (Turner, 1970). In general sero-
groups do not currently have official standing and many strains have been
elevated to serotype standings. Pathogenic leptospires are usually shown by

genus, serotype and strain; for an example, _Leptospira canicola_ (Strain Moulton). Note that in this case the serotype is underlined and ranked similar to a species.

II Cultivation of Leptospires

Leptospires are microaerophillic and grow well at a minimum level of oxygen. Since the discovery of the organism by Inada _et al._ in 1916 several types of media have been evolved for the cultivation of leptospires. In general 4 types of media are used: liquid, semi-solid, solid, and chemically defined.

A. Liquid media.

1. _Noguchi medium_ (1912). Noguchi in 1912 developed a medium for the cultivation of the spirochetes of relapsing fever. A piece of sterile fresh rabbit kidney tissue was placed in a sterile test tube, and a few drops of citrated heart blood from an infected rat or mouse were added followed by 15 ml of sterile ascitic or hydrocele fluid. The tubes were covered with paraffin oil and incubated at $37^{o}C$. Inada _et al._ (1916) used the same media for cultivation of newly discovered leptospires with certain modifications. They used guinea pig kidney tissue instead of rabbit kidney tissue. They found that the incubation temperature of $37^{o}C$ recommended by Noguchi as not suitable for growing leptospires; they obtained best growth at $22^{o}C - 25^{o}C$.

2. _Korthof's medium_ (1932). Korthof in 1932 developed a medium containing tryptose, sodium chloride, sodium bicarbonate, potassium chloride, monobasic and dibasic phosphates and 10 percent rabbit serum. Leptospires were found to survive in this media with the addition of 2-3 drops of fresh

blood from guinea pigs for 1-12 years (Turner, 1970). When satisfactory growth was observed the tube was closed with a rubber bung and kept at room temperature.

3. Gardner's medium (1943). Gardner recommended a simple medium of 12 percent rabbit serum in glass distilled water. The initial growth was satisfactory but growth diminished in serial subcultures.

4. Stuart's medium (1946). Stuart introduced a liquid medium containing aspargine, ammonium chloride, magnesium chloride and glycerine. Rabbit serum was added to a final concentration of 10 percent. As a pH indicator phenol red was used but was not necessary for the growth of leptospires. He successfully cultivated L. icterohemorrhagiae, L. gryppotyphosa, and L. canicola. He compared this medium with Korthof's and Fletcher's media and found it to be as good. Stuart's medium was found to have certain advantages over the other two media. There was no precipitation, as was found in the preparation of Korthof's medium. Stuart's medium could be used to grow organisms for serological studies whereas Fletcher's semisolid medium was not suitable for this purpose. The addition of glycerine helped to keep the medium moist while doing serological work and also it was found to have a growth-promoting factor. With Stuart's medium the organisms have to be sub-cultured more frequently than with Fletcher's semisolid medium. Leptospires tend to become avirulent after serial subculturing in all laboratory media (Turner, 1970).

5. Chang's media (1947). Chang developed two media, a semisolid medium with agar and a liquid medium without agar. The basic ingredients of each medium consisted of tryptose, liver extract powder, monobasic and dibasic

phosphates, sodium chloride and horse serum. Abundant growth of leptospires was obtained. He recommended use of his liquid medium for growing organisms for serological work and the semisolid medium for diagnostic purposes. His studies on growth requirements of leptospires revealed that the optimum pH of media was 7.2, serum was found to be essential and oxygen was necessary.

6. Vervoort's medium (1922, 1923). Vervoort developed a medium containing small amount of peptone, inactivated rabbit serum and buffer. He observed that a pH of 7.2 - 7.4 was optimum. This medium was modified later and the ingredients were standardized (Wolff, 1954). The modified Vervoort's medium contained peptone, Ringer's sol, Sorensen's buffer pH 7.2, and 10 percent inactivated rabbit serum. Excellent growth of all kinds of leptospires was reported.

B. Semisolid medium.

1. Fletcher's medium (1928). Fletcher developed a semisolid medium containing peptone, beef extract, sodium chloride, agar and 10 percent rabbit serum. The growth of leptospires in this medium was rapid and denser than in liquid media but it was not suitable for serological work. The leptospires, being microaerophillic, were found to multiply more in the upper 1-2 centimeters of the medium. Macroscopically, growth was indicated by formation of linear discs of turbidity. The organisms remained viable for extended periods in this medium. It was recommended for maintaining stock cultures and transferred at 2-6 month intervals (Turner, 1970).

2. Chang's semisolid medium (1947). Chang's semisolid medium is described with the liquid media.

C. Solid medium.

Cox and Larson (1957) introduced the first solid medium for the cultivation of leptospires. It contained tryptosphosphate broth, 1% agar, and 10% rabbit serum, and 1% sheep hemoglobin. They were successful in growing both pathogenic and non-pathogenic strains of leptospires. The pathogenic strains took 10-15 days and non-pathogenic strains 7-10 days to form colonies. Two types of colonies were described — one smaller and more opaque, the other translucent and larger in diameter. The margins of the colonies were observed to extend down into the media. Successful use of solid media for the cultivation and isolation of leptospires organisms has been reported by several workers (Kirschner and Grahm, 1959; Roth et al., 1961c; Yanagava et al., 1963; Baseman et al., 1966; Cerva, 1967).

D. Chemically defined media.

Greene et al. (1950) developed a semi-synthetic medium containing dialyzed rabbit serum, salts, vitamins, amino acids, purines, and pyrimidine bases. This medium with peptone was found to support the growth of L. canicola through a prolonged period of serial transfers.

Schneiderman et al. (1951) modified Greene's semi-synthetic medium. The modified medium contained 19 amino acids, vitamins, purines and pyrimidines, and sodium and potassium phosphates, sodium, potassium and calcium chloride, and sodium carbonate. The pH of the medium was 7.2 - 7.4. Filter sterilized, dialyzed rabbit serum 0.8 ml was added to each tube containing 4.3 ml of basal medium. They observed that the albumin fraction precipitated at 71% ammonium sulfate saturation had the greatest growth promoting

factor for L. canicola. They reported that ammonium sulfate precipitated horse or sheep albumin was also satisfactory.

Vogel and Hunter (1961) developed a serum-free chemically defined liquid medium. They observed that thiamine and lipids were essential for growth. The serum was replaced by esterified fatty acids (monoolein, monostearin, methyl palmitate, methyl oleate). L. canicola, L. icterohemorrhagiae, and L. pomona were successfully grown.

Ellinghaussen and McCullough (1965) developed a serum-free medium. The serum was replaced by bovine albumin. The medium contained 20% by volume of oleic acid albumin complex (OAC). L. pomona and 13 other serotypes were successfully cultured for 2 years using weekly transfers. They observed that OAC possessed satisfactory leptospiral growth promoting activity. They also studied growth response of L. pomona to Polysorbate 80 which was incorporated in 1% albumin medium. Little growth resulted in absence of Polysorbate 80. The optimum level of Polysorbate 80 was found to be 0.1% and that of albumin 0.15 to 0.5%. They observed that vitamin B_{12} was essential for the growth of leptospires. This medium was found to be especially adaptable for bacterin production. The advantages of bovine albumin over homologous serum included low cost, ready availability and less anaphylactoid characteristics of the bacterins for cattle. This medium was further modified by Johnson and Harris (1967).

Shenberg (1967) developed a protein-free chemically defined medium for the cultivation of pathogenic leptospires. The medium contained Tween 80, Tween 60, vitamin B_{12}, L-aspargine and thiamine. She also observed that vitamin B_{12} was an absolute requirement in a chemically defined medium.

In all media where serum was used, rabbit serum was found to be most suitable. Noguchi (1918b) found that sera of rabbit, horse and goat were more

suitable than those of guinea pig, sheep, donkey or calf. Rabbit serum contains the highest concentration of bound vitamin B_{12} which is now known to be essential for the multiplication of leptospires (Turner, 1970).

In addition to the different types of media discussed above, cultivation of leptospires in developing chick embryos has been reported (Morrow et al., 1938; Davis, 1939).

III Epidemiology of Leptospirosis

The occurrence and spread of leptospirosis is governed by the general principles of epidemiology. They include reservoir hosts, source of infection, modes of transmission, survival of pathogen (leptospires) in nature (Schwabe, 1969). All pathogenic leptospiroses are believed to be zoonoses, being transmitted from animals to man; as such, the understanding of the nature, habits, and habitat of leptospires is necessary. It was considered for many years that rodents were the principal reservoir hosts. Subsequent investigations revealed the wide range of hosts both domestic and wild animals, birds, arthropod vectors, amphibians and reptiles (Gsell, 1953; Babudieri, 1958; Steele, 1960; Galton et al., 1962; Alexander et al., 1970). In view of the serotype multiplicity and wide range of hosts it is highly unlikely that leptospirosis can be eradicated from domestic and wild life. It is a disease that will remain a problem for public and animal health for years to come (Steele, 1960; Roth, 1964).

A. Reservoir hosts.

Natural reservoirs of leptospires are various species of wild and domestic animals. In addition to the above, isolation of leptospires from amphibians, reptiles, birds and arthropod vectors have been reported.

Babudieri (1958) considered that an obscure phenomenon of biological affinity
existed by virtue of which a state of biological equilibrium was established
between some serotypes of leptospires and some species of animals. In nature
more than one serotype in the same host, or one serotype in many hosts, may be
found (Anon., 1967a).

B. Source of infection.

The principal source of infection is the urine of a carrier (Stoenner,
1957; Galton et al., 1958a, 1962; Babudieri, 1958; Hoeden, 1964; Turner, 1967).
Babudieri (1958) distinguished between the temporary carrier and the true
carrier. He defined a temporary carrier as one which is suffering or is in a
convalescent state during which leptospiruria is limited; the true carrier, on
the other hand, is one which has suffered but has remained a shedder over a
long period of time without clinical signs of illness. It is the latter type
which constitutes a major source of infection (Gsell, 1953). The kidneys are
the principal route of excretion of leptospires. In addition to urine, isola-
tion of leptospires from feces, milk and aqueous humor of the eye has been
reported (Baker, 1948; Howell et al., 1969; Morter et al., 1969). There was
no evidence of excretion of leptospires in saliva though leptospirosis due to
bites of infected animals has been reported. It was suggested that saliva of
the dog might become contaminated because of its habit of licking genitalia or
urine (Hoeden, 1958; Turner, 1967, 1969). Since pathogenic leptospires are not
able to adapt to a saprophytic life, a continuous source of infection is
necessary for the spread of the disease (Gsell, 1953; Babudieri, 1958).

The carrier condition of leptospirosis was recognized early in the
history. Soon after the discovery of the infective agent by Inada et al. in
1916, the search began for the detection of leptospirosis in various species

of animals. Noguchi (1917) isolated L. icterohemorrhagiae from kidneys of wild rats in New York City. Ido et al. (1917) reported that the rat was the carrier of L. icterohemorrhagiae in Japan. They isolated the organism from 37% of common house rats surveyed.

Schmid and Giovanella (1947) observed leptospires in urine of pigs 14-17 days after experimental infection and they were found to persist for 6 months to one year after infection.

Brunner and Meyer (1949) induced a shedder state in dogs by inoculating intraperitoneally 50 million leptospires per kg body weight. The dogs remained urinary shedders for 2-6 months.

McIntyre and Seiler (1953) reported dog shedders for 4½ years. Larson (1953) isolated L. icterohemorrhagica from a large number of rats, and L. canicola from dogs in Washington, D. C. He reported that 18 species of rodents were carriers of leptospires. Bloom (1953) reported rats were shedders of L. icterohemorrhagica and that leptospiruria could persist in dogs for 6 months or longer.

Reinhard (1953a) reported that cattle recovered from the acute stage may remain shedders for 6-8 weeks.

Burnstein and Baker (1954) observed L. pomona in urine of experimentally infected pigs on the 12th day and they persisted for 159 days. Smith and Self (1955) experimentally infected rats by inoculating 0.5 ml of L. australis culture intraperitoneally. Leptospires were first seen in the urine on the 10th day and persisted up to the 77th day when the observations were discontinued.

Hoeden (1955) found the jackal to be a shedder of L. canicola in Israel. Alexander et al. (1957) reported a survey on 1,017 dogs obtained from all parts of the country (U.S.A.). They isolated L. canicola from the urine of 8 dogs, some of which were intermittent shedders. They observed that

leptospiruria was more frequent in animals with a titer of 1:400 or more. Similar findings were reported by Turner (1967). As an exception, isolation of L. pomona from the urine of a dog in absence of serological evidence was reported (Murphy et al., 1958).

Babudieri (1958) in his discussion on animal reservoirs of leptospirosis listed several species of animals which have been reported as carriers of leptospires: rats in Alaska, bats in Andaman Islands and Central Africa, jackals in Israel and cats in Indonesia. Pigs were reported to be carriers for L. pomona and L. hyos for up to one year, cattle for 30-100 days, and in New Zealand, sheep for 9 months. Turner et al. (1958) and Mitchell et al. (1966) reported the isolation of L. canicola from the urine of a new-born calf and piglets, respectively.

Morter et al. (1959) reported isolation of L. pomona from urine of 2 dogs kept on the same premises with cattle having detectable titers.

Gillespie (1963) reported the isolation of leptospires from surface waters in areas occupied by infected cattle. He observed that leptospires were shed in urine for several months and were not materially reduced by vaccination. It has been reported that vaccinated cattle are incompletely protected against infection and may become shedders (Anon., 1967b).

Roth et al. (1963) studied the duration of leptospiruria in naturally infected skunks with L. hyos, L. pomona, L. canicola, L. icterohemorrhagica. The duration of leptospiruria observed with the above strains was up to 774 days, 321 days, 400 days, and 167 days, respectively. They suggested that in nature the duration may be still longer, as many of the animals died due to other causes during the study.

Low (1964) reported that dogs normally shed leptospires in urine for 6-18 months, but occasionally are found to shed the organisms for 3 or more years.

Aragon et al. (1965) isolated 4 strains of leptospires from field rats. Eight percent of the rats surveyed were found to harbor the organisms. Mitchell et al. (1966) reported that leptospiruria in pigs might extend from 2 weeks to over 2 years and may be intermittent.

Hubbert and Shotts (1966) isolated L. canicola from the urine of 10 of 19 apparently healthy dogs in a kennel. While studying the effect of various antibiotics on shedders they found that streptomycin was effective at least up to 22 days post treatment. Other antibiotics were temporarily effective. The dogs returned to shedder states in 6-63 days post treatment. They suggested that leptospires may remain intracellular or in interstitial tissue where antibiotic concentrations are not high enough. Babudieri (1958) observed leptospires to colonize in renal tubules and appear in urine when conditions were favorable. Animal carriers were often found to excrete 10^8 organisms per ml of urine (Anon., 1967a).

Imbabi et al. (1967) observed leptospires in the urine of 7 experimentally infected calves from the 13th to the 37th day.

Michna and Campbell (1969, 1970) observed that leptospiruria was evident 2-3 weeks after the onset of the disease and could persist up to 2 years, in some cases being intermittent. They conducted a survey of wild life in northeast and southwest Scotland and found that many species of wild life were carriers.

Hanson et al. (1971) observed leptospires in the urine of an experimentally infected pig on the 12th day that persisted up to 40 days when observations were discontinued.

Baker and Little (1948) isolated spirochetes from the milk of clinically ill cows which was infective to guinea pigs, rabbits, mice, and embryonated eggs, and in normal cows by subcutaneous and intranasal routes. The organism was also found in blood and urine.

Morter et al. (1969) isolated L. pomona from the aqueous humor of the eyes of a horse suffering from periodic opthalmia.

Turner (1967) has cited reports of survival of leptospires in the intestinal tract of certain flies which may be deposited in their feces. Babudieri (1958) reported the isolation of leptospires from feces of wading birds in Italian rice fields for 26 days after experimental oral infection.

Hoeden (1958) has cited the observations of Fuhner (1950) according to which human beings seldom excrete leptospires for more than 4 weeks, but exceptionally might be shed for 11 months as reported by Johnson (1950). Spinu et al. (1963) found that convalescent human patients may excrete leptospires for up to 190 days. Taylor and Goyle (1931) considered the possibility of the spread of leptospirosis due to contamination of water with infective human urine. Gsell (1953) failed to detect leptospires in urine of the majority of the human cases which he studied. He reported detection of leptospires in one case after the 64th day.

C. Transmission.

Transmission of leptospirosis from an infected animal to a susceptible host depends on two factors: (1) Route of infection; and (2) Modes of transmission.

1. Route of infection. Leptospires can enter the body of a susceptible host through the skin or mucous membranes of the various parts of

the body such as eyes, nasopharynx, and mouth, or alimentary canal (Inada et al., 1916; Thiel, 1948; Thiel and Engelbrecht, 1957; Alston and Broom, 1958; McCrumb, 1957; Galton et al., 1958a, 1962; Hoeden, 1964; Turner, 1967; Alexander et al., 1970).

a. Skin. Inada et al. (1916) conducted experiments in guinea pigs to study the route of infection through the skin. A liver emulsion containing L. icterohemorrhagica was applied to the abdominal wall which was then shaved with and without injury to the skin. Eighty-six and 77% of the guinea pigs became infected, respectively. They concluded that the spirochete was able to penetrate macroscopically healthy skin. Their observations on 55 clinical human cases revealed that infection could take place through intact skin. Bloom (1953) while discussing the epidemiology of canine leptospirosis stated that one of the possible routes of infection in dogs was a fine delicate skin in interdigital space. Gsell (1953) reported that leptospires could penetrate the skin more easily if it were abraded.

Thiel and Engelbrecht (1957) were able to induce infection in human beings through abraded skin by applying cultures of an avirulent strains of L. grippotyphosa and L. icterohemorrhagica, whereas they failed to do so with intact skin. In earlier studies (Thiel, 1948) he failed to induce infection through the undamaged skin by contact with diluted urine of a carrier. Bryan (1957) reported that leptospires could enter the body through cuts and abrasions of the skin.

Coghlan et al. (1957) successfully infected pigs by subcutaneous inoculation and through scarified skin, but failed to do so by intranasal inoculation. They suggested that natural infection could take place through contamination of wounds.

Steele (1958, 1960) in his review of epidemiological aspects of leptospirosis stated that abraded skin was one of the portals of entry of leptospires. Borg Petersen (1944) considered that a wet or sodden state of skin, though not visibly injured, was favorable for penetration of leptospires. It has been reported by others that leptospires could enter abraded skin and possibly through unbroken skin if it were first softened by long exposure to water (Babudieri, 1953; Galton et al., 1962; Low, 1964; Anon., 1967a; Turner, 1969; Alexander et al., 1970).

b. Mucous membrane. Infection through the mucous membranes of the eyes, nasopharynx and mouth have been reported (vide supra).

c. Alimentary canal. Infection through the alimentary tract has been reported, although some workers have failed. Inada et al. (1916) believed that infection in European Weil's disease occurred through the alimentary canal. They were also successful in inducing infection in guinea pigs either by giving enemas or by feeding 2 grams of infective liver emulsion. Baker and Little (1948) suggested the possibility of aerosol infection, whereas Babudieri (1953) considered infection by ingestion or inhalation as an exception.

Burnstein and Baker (1954) were successful in infecting pigs with L. pomona by intranasal contact or subcutaneous inoculation but could not infect pigs per os.

Thiel and Engelbrecht (1957) exposed 8 guinea pigs to drinking water containing a virulent culture of L. icterohemorrhagica but only one became infected. Similar results were also obtained with sewer rats. They could not induce infection in man by administering an avirulent strain in gelatin capsules or by bringing organisms in contact with nasal or buccal mucous

membranes. They were able to induce infection in guinea pigs by intranasal inoculations. Their findings suggested that virulent strains might possess greater power of penetration than avirulent ones.

2. <u>Modes of transmission</u>. Transmission may be direct or indirect.

a. Direct. Direct transmission, though rare, has been reported following bite wounds of dogs and rats (Turner, 1967), venereal (Michna, 1969; Turner, 1969), transplacental (Podgwaite et al., 1955; Bridges, 1958; Turner et al., 1958; Fennestad and Borg Petersen, 1958; Chung huei Lan, 1963; Mitchell et al., 1966; Manrique and Roberts, 1968; Turner, 1969; Hanson et al., 1971) and predation (Reily et al., 1970).

b. Indirect. Indirectly the disease may be transmitted by contact with water, soil or sewage contaminated with the urine of a shedder (Ido et al., 1917; Sawyer and Bauer, 1928; Alston and Broom, 1958; Turner et al., 1958; Babudieri, 1958; Hoeden, 1958, 1964; Anon., 1965; Turner, 1967, 1969; Manrique and Roberts, 1968; Alexander et al., 1970).

D. <u>Survival of leptospires in nature.</u>

Successful survival of pathogens outside the body of the host is one of the contributing factors in spread of many diseases. Pathogenic leptospires survive in nature for relatively short periods of time and are not able to adapt to a saprophytic life (Gsell, 1953; Babudieri, 1958). The leptospires are susceptible to heat, desication, sunlight, excessively high or low pH, high salt concentrations, chemical disinfectants and putrefaction. The conditions favorable for survival are high humidity, slightly alkaline reaction, environmental temperature of 22°C or above, and the presence of organic

substances (Gsell, 1953; Galton et al., 1958a; Babudieri, 1958; Steele, 1960; Alexander et al., 1970).

The survival of leptospires in urine, feces, water, sewage, sea water, soil, animal tissues, semen and milk, and the effect of temperature, pH, moisture, and contaminants have been studied.

1. Urine. Survival of leptospires in urine is closely related to the pH and antibody content in urine (Morse et al., 1958). Noguchi (1918a) observed that L. icterohemorrhagica survived for at least 24 hours in slightly alkaline urine, but not in acidic or moderately alkaline urine.

Sawyer and Bauer (1928) failed to isolate leptospires from human urine 3 hours after inoculation of urine with leptospires culture.

Fuhner (1950) found that leptospires survived for 5 hours in rats' acidic urine, and for 24 hours in neutral or alkaline urine.

Borg Petersen (1953) warned against the belief that leptospires do not survive in acidic urine. He reported that leptospires survived for ½-2 hours at pH 5 - 5.5. He suggested that they might survive longer at pH 6.

Kirschner and Maguire (1957) reported survival of L. pomona in undiluted bovine urine for 30-90 minutes, for 22 days in 1:5 dilution, and for 42-63 days in urine diluted with tap water 1:10 to 1:100.

2. Feces. Noguchi (1918a) observed that leptospires disappeared in 24 hours in human and guinea pig feces. Sawyer and Bauer (1928) failed to recover leptospires from human feces 3 hours after the inoculation with cultures.

3. <u>Water</u>. Noguchi (1918a) observed that <u>L</u>. <u>icterohemorrhagica</u> did not survive for more than 2 days in river water and 6 days in distilled water exposed to air.

Sawyer and Bauer (1928) found that <u>L</u>. <u>icterohemorrhagica</u> survived for 55 days in stagnant water and non-pathogenic leptospires for up to 115 days. Thiel (1937) reported survival of leptospires in water for 22 days without changes in virulence. Chang <u>et</u> <u>al</u>. (1948) reported survival of <u>L</u>. <u>ictero-</u> <u>hemorrhagica</u> for 18-20 days in tap water exposed to air, with pH 7.3 to 7.5, 5-6 days in river water and 18-20 hours in sea water.

Kirschner and Maguire (1957) studied the survival of L. pomona in rain water and sea water. In sterile rain water the survival period was 21-42 days, and 12-18 days when contaminated with other bacteria. In undiluted sea water the survival period was 18-24 hours, 10 days and 35 days when diluted 1:5 and 1:10 to 1:100, respectively.

Okazaki and Ringen (1957) found <u>L</u>. <u>pomona</u> to survive for 3-6 days in stagnant water. Shutyaev (1959) observed that a leptospires serotype identical with <u>L</u>. <u>grippotyphosa</u> survived in water for 336 days without change in pathogenicity for puppies and guinea pigs. After 976 days it still produced a high serological titer in rabbits. A few other strains were found to remain virulent after 1,318 days in water.

Gillespie (1963) isolated <u>L</u>. <u>pomona</u> from surface water in areas occupied by infected cattle. The organism was found to persist for 16 days. He observed that <u>L</u>. <u>pomona</u> could survive for a week or more in water having neutral or alkaline reaction whereas they did not survive in contaminated water.

4. _Sewage_. Noguchi (1918a) observed that L. icterohemorrhagica did not survive in sewage for more than 48 hours. Chang _et al_. (1948) found L. icterohemorrhagica could survive 12-14 hours in domestic sewage and 3-8 days in diluted sewage. Kirschner and Maguire (1957) studied the survival of L. pomona in abattoir sewage. In raw undiluted sewage it was found to survive for 12-14 hours, 10 days when diluted 1:10 to 1:100. In Seitz filtered undiluted sewage the survival period was 30 days, and 40 to 90 days when diluted 1:10 to 1:100.

5. _Soil_. Noguchi (1918a) found L. icterohemorrhagica to survive for not more than 72 hours in soil with neutral pH and for not more than 24 hours in the presence of contaminants.

Smith and Self (1955) conducted experiments to study the survival of L. australis in soil with different ranges of moisture and pH. They observed that in soil containing sufficient moisture the organisms were viable for 46 days with pH of 6.2. The pH of water obtained from soil ranged from 6.6 to 6.9 at the time of inoculation into two guinea pigs. In soil contaminated with rats' urine leptospires were found to be viable for 19 days. They concluded from their experiments that L. australis excreted by a rodent carrier might survive in soil with sufficient moisture for at least 15 days. The survival of the organism in soil for 46 days was attributed to the addition of medium in culture. They also observed that excessive amount of urine had an adverse effect on survival. They suggested that surface water becomes infected by migration of leptospires from soil previously contaminated by a shedder, after rain has fallen.

Okazaki and Ringen (1957) observed that in dry soil the organisms were found to be dead within 30 minutes by darkfield microscopy and cultural

attempts were unsuccessful after 2½ hours. In damp and supersaturated soil the organisms were found to survive for 5 days and 193 days, respectively.

Kirschner and Maguire (1957) found that L. pomona survived for 1-2 weeks and 3 weeks in supernatant of jars containing soil covered by urine containing leptospires diluted 1:10 and 1:100, respectively.

Babudieri (1958) while discussing the epidemiology of leptospirosis in Italian rice fields found that in rice fields the environmental conditions of semi-stagnant tepid water, infestation by carrier rodents and stretches of mud left by the rivers were favorable for the survival of leptospires. He also believed that some species of leptospires, less sensitive than L. ictero-hemorrhagica, not only survive longer in water but could also multiply.

Smith and Turner (1961) found leptospires to survive for 3-7 days in soil with pH range of 3.7 to 7.3.

6. Animal tissues. Noguchi (1919) observed that L. icteroides degenerated within 12 hours in liver and kidney tissues from an infected guinea pig stored at 10°C.

Buchanan (1927) reported that tissues of an infected guinea pig were infective for 26 days when kept in refrigerator.

Bernkof et al. (1948) obtained inconsistent results with tissues of infected bovine. In their first experiment fresh liver tissue from a jaundiced animal was non-infective after storage for 24 hours in a refrigerator even though leptospires could be seen microscopically. In a second experiment kidney and liver tissues were removed 5 hours after death and stored in a refrigerator for 12-28 hours. A 10% suspension of these tissues was infective to 4 inoculated calves. In the third experiment a 10% suspension of liver and gluteal muscle from a freshly slaughtered infected animal was not

infective. They concluded from the above experiments that in certain cases tissues may remain infective for 33 hours after death.

Mantovani (1950) reported that a piece of diaphragm of a cow infected with L. icterohemorrhagica was infective for guinea pigs following storage at -28°C to +2.8°C for 3 days.

O'Connel and Broom (1952) isolated L. icterohemorrhagica from the kidney of a rat which had been stored for 3 days in refrigerator at 5°C. Bryan et al. (1953) reported the first isolation in the U.S.A. of L. pomona from swine fetuses. They used saline suspensions of liver and kidney to infect guinea pigs.

Kotova (1955) studied survival of leptospires in meat of infected sousilk carcasses. They were inoculated with 0.2 ml of a leptospires culture and killed at 1-7 day intervals after infection. The meat from infected carcasses was infective for susceptible sousilks up to 24 hours after death. Tissues stored at 6°C for 48 hours were infective to 30% of animals inoculated. Meat dried over 8 days to 75% of its original weight remained infective. After drying for 13 days only 15% of the animals inoculated became infected. Tissues were not infective following storage for 10 days at -10°C to -20°C.

Alston and Broom (1958) reported that survival period of organism in infected animal tissue was influenced by postmortem pH changes. They suggested that in cases of infection due to direct contact with the postmortem tissues the organism might possess a greater virulence than primary cultures obtained from tissues and grown on laboratory media prior to inoculation.

Michna (1959) reported the isolation of L. canicola from a kidney of an infected pig up to 5 hours after death. Motile L. canicola were demonstrated for 7 days after death in films of fluid prepared after maceration of a

naturally infected pig kidney stored at 0-4°C. On the 8th and 9th day only non-motile forms were seen. The organisms were found to be active for 3 days when kept at room temperature after being previously stored for 7 days at 0-4°C. Motility was inhibited at a tissue pH of 5.9, and disappeared within 24-48 hours at pH 5.8 or lower. On the 2nd and 5th day of storage at 0-4°C the pH of the tissue was 6.73 and from such material leptospires were recovered for up to 12 days after death.

7. Semen. Jones (1958) found that L. pomona survived in semen diluent without antibiotics for at least 30 days after freezing and storage at -190°C and was infective to guinea pigs. A serologic response was observed in guinea pigs inoculated with semen stored for 108 days. He suggested that leptospires could survive for at least 108 days. No survival was observed when antibiotics were added.

8. Milk. Bernkof et al. (1948) found leptospires survived in milk for 3 days when stored under refrigeration without the loss of virulence. Kirschner and Maguire (1957) in their earlier studies had found that undiluted milk contained an inhibitory factor which was lethal to leptospires. Further investigation of milk diluted with tap water revealed that leptospires could survive for 7-9 weeks. If the diluted milk was contaminated by other micro-organisms leptospires survived for only 1-2 days. Addition of tap water to contaminated milk prolonged the survival time up to 2-3 weeks. Mitchell and Boulanger (1959) reported the possibility of the transmission of leptospirosis through contaminated milking equipment. Turner (1969) reported that lepto-spires could survive only for a short time in milk and were destroyed by heating to 60°C.

9. Effect of pH, temperature, moisture, and contaminants.

a. pH. Noguchi (1918a) found that a slightly alkaline pH was favorable for the survival of leptospires. Very high or low pH levels were detrimental to L. icterohemorrhagica.

Chang et al. (1948) reported that a pH of 7.3 to 7.5 allowed survival of L. icterohemorrhagica for 18 days in plain tap water. Burnstein and Baker (1954) reported that in pigs' urine with an acidic pH the organisms were very active.

Smith and Self (1955) reported survival of L. australis for 19 days in soil with pH 6.6 to 6.9.

Gsell (1953) stated that an alkaline reaction of urine was essential for survival of leptospires. In acid urine they had already perished in the urinary bladder.

Okazaki and Ringen (1957) found that the critical level of pH for the survival of L. pomona in nature was between 6 and 8.4. The organisms were found to survive for a longer period at a lower pH range when stored at $7-10^{o}C$ and at a higher pH range when stored at $20-26^{o}C$.

Kirschner and Maguire (1957) found that L. pomona survived only for 30-90 minutes in cows' undiluted urine with pH levels of 6.3 to 7.2 and for 30 minutes with pH 8 - 8.1.

Babudieri (1958) reported that optimum pH level for the survival of leptospires for long periods was between 6.35 to 7.96. However leptospires were found to survive for 6 days at extreme pH levels of 6.24 and 8.23.

Smith and Turner (1961) studied the effect of a pH range of 5.3 to 8 on the survival period of 4 strains of leptospires (L. icterohemorrhagica,

L. hyos, L. australis and L. javanica). They reported that at pH $<$ 7 the survival period was 10-117 days and 21-152 days at pH $>$ 7.

Gillespie (1963) while isolating L. pomona from surface waters in areas occupied by infected cattle observed that the organism survived for a week or more with neutral or alkaline reaction.

Turner (1967) reported that pH values outside the range of 6.2 to 8 were unfavorable for the survival of leptospires.

Alexander et al. (1970) recommended adjustment of pH of urine to 7.2 to 7.6 for successful isolation of leptospires.

b. Temperature. Chang et al. (1948) studied the effect of temperature on survival of leptospires under different conditions. In sterile tap water at 25 to 27°C leptospires survived for 30-32 days and for 3-4 days in 10% sewage in tap water. Okazaki and Ringen (1957) reported the optimum range of temperature for the survival of L. pomona lies between 7°C and 26°C. Temperatures below 7 and above 34°C were lethal.

c. Moisture. Smith and Self (1955) found L. australis to survive for 46 days in soil with sufficient moisture. Okazaki and Ringen (1957) found that L. pomona did not survive for more than 30 minutes in dry soil, whereas in damp soil it was found to survive for 5 days and for 193 days in super-saturated soil. Desication has been reported to be lethal to all spirochetes (Turner, 1967; Galton et al., 1962; Hoeden, 1964).

d. Contaminants. Bacterial contamination was found to be lethal to leptospires (Noguchi, 1918a; Chang et al., 1948; Kirschner and Grahm, 1959; Gillespie, 1963; Hoeden, 1964).

E. Zoonoses.

All leptospiroses are considered zoonoses (Gsell, 1953; Galton et al.,
1962). Leptospirosis is the world's most widespread contemporary zoonosis
(Hoeden, 1964). Man is an accidental host for leptospires and is a dead end
in the chain of infection (Galton et al., 1962; Turner, 1967; Alexander et al.,
1970). Infection in man is usually due to indirect contact with contaminated
material. Persons who are exposed to such material in daily activities are
more liable to be infected; as such it is considered an occupational hazard
(Hoeden, 1964; Alexander et al., 1970). Leptospiral infection in man has been
reported from all parts of the world.

Ido et al. (1917) observed leptospirosis in cooks, maids, pastry cooks,
bone meal manufacturers, vegetable dealers and coal miners in Japan who had a
chance to come in contact with infected rats. Wadsworth et al. (1922)
reported the isolation of L. icterohaemorrhagiae from a laboratory worker who
was associated with the investigation of the epidemic of infectious jaundice
in Albany, N. Y. The blood was negative for darkfield examination but guinea
pig inoculation was positive. This was the first human case reported in the
U.S.A.

Meyer et al. (1938) reported leptospirosis in two veterinarians who
were caring for infected dogs. L. canicola was isolated from the urine of one
of them.

Havens et al. (1941) stated that in England leptospirosis was found
most often in fish handlers, coal miners, and sewer workers who were working
in places infested with rats. Bathing in water polluted with rats' urine was
one of the principal modes of transmission to human beings. In 1939 they
investigated an outbreak of leptospirosis in Philadelphia. Seven people were

infected by bathing in a public pool which was adjacent to cattle barns and refuse pits infested with rats.

Stiles and Sawyer (1942) analyzed the reports of 78 cases of leptospirosis which occurred between 1905 and 1940. They considered leptospirosis as an occupational hazard where the occupation exposed the patient to carrier rats, dogs or to moist materials contaminated by the urine of such animals. Gardner (1946) conducted a serological survey in human beings from 1940-1945. Sixteen percent were found to be positive. The incidence was found to be 18% in coal handlers, 6.8% in farm workers, 4% in sewer workers. Positive cases were also found to a lesser extent in butchers, fish workers, army and navy personnel. He observed that the incidence was highest in the summer; this he attributed to bathing in water polluted with rats' urine.

Schaeffer (1951) attributed an outbreak of leptospirosis in Geneva, Alabama, in 1950 to swimming in a pool where 50 persons were infected. There was a serological evidence of leptospirosis (L. pomona) in pigs, cattle, horses, and mules in the area.

Haunz and Cardy (1952) reported 9 cases of canicola fever in one family. The source of infection was a pet dog carrier. The dog had been observed catching rats.

Gochenour et al. (1952) investigated an outbreak of Fort Bragg fever or peritibial fever which had occurred in troops in North Carolina in 1942. His investigations revealed that Fort Bragg fever was leptospirosis caused by L. autumnalis. The organism was recovered by hamster and guinea pig inoculation.

Kirschner (1953) in his comments on the paper presented by Little and Raker (1953) mentioned that in New Zealand the leptospirosis in human beings was associated with pigs and dairy cattle.

Babudieri (1953) in his studies on leptospirosis in workers in Italian rice fields observed that the source of infection was field rats and mice. A serological survey revealed 20.5% of workers and 25% of mice being positive. The percentage of infection was found to be highest, up to 80%, in persons working for more than 20 years in rice fields.

Cockburn et al. (1954) attributed an outbreak of leptospirosis to swimming in a pool. A survey of the domestic animals of the area revealed titers against L. canicola in horses and dogs. Williams et al. (1956) investigated canicola fever outbreak in man. The source of infection was found to be a small swimming hole. A serological survey was conducted on farm animals which included cattle, horses, goats, pigs, mules and dogs. All species except goats had a titer against L. canicola. The L. canicola was isolated from blood and urine of human beings, from urine of dogs, and from kidney emulsions of swine. This was the first report of the isolation of L. canicola from swine.

Hoeden (1956) reported leptospirosis in 8 people who were handling pigs. A jackal was considered as the original source of infection.

Varfolomeva (1958) reported an outbreak of leptospirosis in Russia in 1952 due to swimming in a river contaminated with rats' and pigs' urine. An organism closely related to L. canicola was isolated from blood and urine of human patients, and from kidneys of rats.

Galton et al. (1958a) in their report on epidemiological pattern of leptospirosis listed several outbreaks of leptospirosis which occurred in the U.S.A. between 1940 and 1952. All the outbreaks were attributed to swimming in contaminated water. The source of infection was urine from carrier dogs, swine and cattle. They considered leptospirosis an occupational hazard and have referred to outbreaks of leptospirosis in various parts of the world

occurring in abattoir workers, cane and rice field workers, dairy farmers, animal husbandrymen, trench diggers and veterinarians.

Anon. (1963). Physicians at the State College for Women in Columbus, Mississippi, found 19 students infected with leptospirosis after handling a hamster taken to a dormitory from the school's biology laboratory.

Pertzelan and Pruzanski (1963) reported that the jackal was the principal reservoir of L. canicola in Israel. Cattle and pigs became infected from jackals and men working on these farms became infected from the farm animals.

Hoeden (1964) has quoted several reports of outbreaks of leptospirosis in human beings in various parts of the world which were attributed to drinking of contaminated water. Sturdza et al. (1966) reported two human cases of leptospirosis due to handling of raw kidneys from pigs. A serological survey conducted on abattoir workers revealed 37% and 16% positive cases who were handling pigs and cattle, respectively. Sixty-five percent of pigs and 30% of cattle were also positive serologically.

Lawson (1966) investigated 10 cases of canicola fever in man which occurred in Glasgow, England, between 1957 and 1963. The source of infection was pigs with which the patients had contact. L. canicola was isolated from kidneys of pigs and rats and also from a nearby pond. He considered the canicola fever as an occupational disease occurring in piggery workers.

Crawford et al. (1969) reported that out of 43 cases of leptospirosis diagnosed serologically in Iowa, 39 cases (90%) were associated with meat processing, handling of livestock and hunting. The cases included 2 veterinarians who were meat inspectors and a microbiologist who was engaged in vaccine production. Two cases were associated with swimming in contaminated water. They suggested that the most probable source of exposure was cattle,

swine, squirrels, raccoons, dogs, and water contaminated with urine of infected animals.

Diesch et al. (1969) attributed an outbreak of leptospirosis in Iowa to swimming in a contaminated creek. L. pomona was isolated from creek water and 30.5% of cattle had a titer against L. pomona. Sakula and Moore (1969) reported 4 cases of leptospirosis in herdsmen in Surrey, England, with aseptic meningitis.

Turner (1969) considered leptospirosis as an occupational hazard, though its incidence has decreased. The professions still considered risky were agriculture (farm work, market gardening), animal contacts (veterinarians, livestock attendants, kennel personnel, rodent examiners), meat handlers (abattoirs, meat packing and processing), construction workers (roads, canals, drains, gravel pits), forestry, surveying, military exercises, recreation (swimming, camping, fishing, boating), and sewer workers when hygienic conditions failed.

Bigler et al. (1970) reported that in Florida most of the cases of leptospirosis in human beings had a history of contact with dogs, rats, cattle, or pigs.

Zack et al. (1971) reported a case of leptospirosis in New York City in which the source of infection was considered to be water contaminated with rats' urine.

IV Laboratory Diagnosis of Leptospirosis

Establishment of definitive diagnosis is a prerequisite for effective treatment and control of leptospirosis. Reinhard (1953b) stated that serology is the most useful laboratory tool for confirmation of the diagnosis of leptospirosis, other methods being unreliable or not practical. Gochenour (1957)

reported that demonstration and recovery of leptospires from body fluids and tissues affords the most definitive confirmation of the diagnosis, especially in acute and fatal cases. Diagnosis based on clinical signs is unreliable as it frequently results in misdiagnosis (Turner, 1967).

There are many conditions which exhibit one or more of the clinical signs observed in leptospirosis. Alston and Broom (1958), Galton et al. (1962), and Turner (1967, 1969) suggested that leptospirosis should be considered in the differential diagnosis of influenza-like illness, aseptic meningitis, encephalitis, non-paralytic poliomyelitis, rickettsiosis, dengue-like illness, enteric illnesses, brucellosis, jaundice with fever (yellow fever and infectious hepatitis), and pyrexia of unknown origin.

Reliable laboratory procedures that will provide the earliest confirmation of the diagnosis of leptospirosis are highly desirable. The reliability of any laboratory procedure largely depends on its application at the proper time in the course of the disease. The diagnosis of leptospirosis is not difficult if suitable specimens are collected at the proper time and submitted to the laboratory for examination (Galton et al., 1962; Turner, 1967, 1968; Dennis, 1969).

In recent years there has been an increasing awareness of the prevalence of leptospirosis in man, domestic animals, and wild life. Leptospirosis has been found in many countries, such as Malaya and Ceylon, where its prevalence was previously unsuspected. This has resulted in an increased demand for the laboratory diagnosis of leptospirosis (Gochenour, 1958; Turner, 1968, 1970).

A. <u>Demonstration of leptospires in body fluids and tissues.</u>

1. <u>Darkfield examination of blood and urine.</u> Leptospires are not
visible through the optical microscope on a light field but show up quite
brightly under darkfield (Babudieri, 1961). Blood, cerebrospinal fluid,
aqueous humor from the eyes, urine, and tissue emulsions may be examined at
certain phases during the course of the disease by darkfield for the detection
of viable leptospires. Gochenour (1953) in his attempts to demonstrate lepto-
spires in human blood found the darkfield examination of no value. False
positive diagnoses were made due to the presence of pseudospirochetes, proto-
plasmic extrusions of formed blood elements. False negative results were
obtained by darkfield examination of blood which proved positive by other
methods. In view of such false positive and negative results he was of the
opinion that positive diagnosis of leptospirosis cannot be made solely on
darkfield examination.

Wolff (1954) reported that darkfield microscopy is an indispensable
aid in all leptospiral investigations. He recommended examination of blood in
leptospiremic phase. Oxalated blood was centrifuged at 1,500 RPM for 15
minutes. A drop of plasma was examined under darkfield. If this was negative
the plasma was again centrifuged at 10,000 RPM for 20 minutes and the sediment
examined. Of the 100 blood samples examined only 8 were positive by direct
darkfield examination, whereas 24 were positive after double centrifugation.
He cautioned however about false positive diagnosis being made due to the
presence of pseudospirochetes. Darkfield examination of blood was less suc-
cessful when compared with animal inoculation or culture. Galton <u>et al</u>. (1962)
reported that darkfield examination should be used only as ancillary method
and should never be relied upon as the only diagnostic test. Blenden (1964)

reported that when leptospires are found in darkfield examination they have significance but their absence is of no diagnostic value.

Turner (1970) recommended the darkfield examination of blood, cerebrospinal fluid, and liver spleen and kidney emulsions during the leptospiremic phase, and urine in the leptospiruric phase. Alexander et al. (1970) did not recommend direct darkfield examination, particularly of blood, as a single diagnostic procedure as it resulted so frequently in misdiagnosis. It may also result in failure to diagnose because of the low concentration of leptospires in blood and cerebrospinal fluid. They recommended that all darkfield microscopic diagnoses should be confirmed by cultural or serological methods.

Tissue impression smears can be examined by darkfield microscopy. Sturdza et al. (1966) demonstrated leptospires by darkfield examination in a kidney impression smear treated with 10% acetic acid solution for 5-10 minutes. Thick smears were treated with 0.15% solution of trypsin for 1-3 minutes.

2. Staining of leptospires: Giemsa and silver techniques. Tissue impression smears can be stained with Giemsa, or Fontana's silver impregnation method, and tissue sections with Leviditi's silver impregnation and Warthin-Starry method. Leptospires have been demonstrated by staining in aborted fetuses where isolation was not successful (Galton et al., 1962; Turner, 1970). The disadvantages of silver staining methods are, other tissue elements— especially hematogenous pigments, nuclei, and melanin— are also argentophilic; only intact leptospires still retaining their morphological features can be identified.

B. <u>Isolation of leptospires from body fluids.</u>

    1. <u>Direct culture.</u>

        a. Blood. Gochenour <u>et al.</u> (1953) reported isolation of leptospires by direct culture of blood and cerebrospinal fluid during an investigation of leptospirosis in humans in Puerto Rico between 1950 and 1952. They recommended the inoculation of a minimal quantity of blood (.03 ml) into Fletcher's medium immediately after collection. They observed that direct culture of blood was most successful between the 3rd and 9th day of illness. Wolff (1954) successfully isolated leptospires by direct culture of blood obtained during the first 8 days of illness.

        Clark <u>et al.</u> (1960) reported the first isolation of <u>L. pomona</u> from cattle in the U.S.A. by direct blood culture while investigating an outbreak of leptospirosis in Pennsylvania in 1959.

        Galton <u>et al.</u> (1962) recommended culture of blood during the febrile stage before the commencement of the treatment with antibiotics. They also suggested inoculation with minimum quantity of blood as excessive blood was found to have an inhibitory effect. They found this method highly successful in isolation of leptospires from infected dogs.

        Turner (1970) recommended bedside culture of blood obtained as early as possible after the onset of disease. If clotted blood was received in the laboratory the clot was triturated and inoculated. He did not recommend anticoagulants as they were found to be detrimental to viability.

        b. Urine. Examination of urine for the presence of leptospires is indicated after the first week of illness. Direct culture of urine rarely yields positive results due to bacterial contamination. Moreover the shedding

of the leptospires in urine may be intermittent (Wolff, 1954). Another factor interfering in successful culture of urine is the presence of antibodies in urine. Stuart (1956) found that in humans antibodies usually appeared in urine by the 10th day and invariably in the 2nd week. The titers as high as 1:300 were observed. A titer of 1:30 was found to be lethal to leptospires in urine. Dilution of urine with saline to eliminate antibodies was unsatisfactory. With absorption of urine with heat killed leptospires they were able to infect 10 out of 13 guinea pigs, but only in 4 guinea pigs with unabsorbed urine. Similar findings were reported by others (Menges et al., 1961; Roth, 1964).

Menges et al. (1958) reported successful isolation of leptospires from urine by bladder tapping. Immediately after collection 3 drops were inoculated in each of 4 tubes of Fletcher's medium. This method was successfully used on dogs, cats and guinea pigs and was claimed to be more efficient than animal inoculation.

White and Ristic (1959) reported the isolation of L. pomona from urine of infected guinea pigs and cattle by filtering the urine through Millipore filters of 0.45 u pore size. These filters had been reported earlier to be suitable for the purification of contaminated leptospiral culture (Rittenberg et al., 1958).

Menges et al. (1960) and Menges and Galton (1961) described the method for the isolation of leptospires from voided urine by direct culture. One drop of voided urine was directly inoculated in Fletcher's medium and the remaining portion was serially diluted up 10^{-11} in buffered saline. Media tubes were inoculated with one drop from each dilution. The optimum dilution to obtain maximum percentage of pure cultures was between 10^{-4} and 10^{-7}.

White et al. (1961) reported that when very few leptospires were present bladder tapping was most suitable.

Roth (1961a, 1964) had excellent results with a combination of bladder tapping and dilution methods in isolation of leptospires from urine of dogs and skunks.

Sulzer (1964) used the dilution method to isolate L. icterohaemorrhagiae, strain hardjo, from urine of naturally infected dairy cattle.

Gale et al. (1966) and Hubbert and Shotts (1966) applied the dilution method and bladder tapping for isolation of leptospires in humans and dogs.

Alexander et al. (1970) commented that successful application of dilution method is dependent upon the leptospires being in greater number than contaminants.

c. Tissues. Galton et al. (1962) recommended that necropsy should be done as soon as possible after death to avoid invasion of tissues by contaminants. Small animals were dipped in 10% cresol solution for 10-15 minutes and large animals were thoroughly swabbed with the solution. A 10% suspension of kidney in buffered saline was serially diluted up to 10^{-6} and 3 drops from each dilution were inoculated into Fletcher's medium and 1 drop on Cox's solid medium.

Roth (1964) inoculated 10% kidney suspensions in buffered saline into Stuart's medium to isolate leptospires from skunks. He reported that kidney suspensions gave more positive results than urine.

Turner (1970) suggested 3 methods to culture tissues:

(1) A 10% suspension of the tissue is serially diluted to give 10-fold dilutions and 2-3 drops from each dilution are inoculated into medium. Higher dilutions were found to allow successful culture by reducing the inhibitory

effects of lipids and contaminants present in the tissue.

(2) The surface of the organ is first seared with a hot spatula. A sterile Pasteur pipette is inserted through the seared portion into the tissues to obtain a small sample and directly inoculated into culture medium.

(3) The tissue is expressed through a 2 ml syringe without needle and inoculated directly into culture medium.

Liver, spleen and kidney tissues from animals dead during leptospiremic phase and the cortex of the kidney from animals which had survived for 21 days or more were recommended.

2. _Animal inoculation_. Ringen and Okazaki (1956) reported that guinea pigs and white mice were equally susceptible to _L. pomona_. The hamster was resistant, and 1-day-old chicks were highly resistant. Fisher _et al._ (1958) found that the hamster was more suitable than the baby chick for isolation of _L. canicola_. Roberts and Turner (1958) noted that chinchillas were more susceptible to _L. pomona_ than were guinea pigs and hamsters. Gochenour _et al._ stated that guinea pigs, golden hamsters, Swiss white mice, meriones, and baby chicks could be used for the isolation of leptospires. Galton (1962) reported that weanling hamster is the animal of choice, however guinea pigs, baby chicks, and gerbils could be used for the isolation of leptospires.

Turner (1970) suggested that animal inoculation for the isolation of leptospires should be done as a supplementary measure to direct culture. Animals at weanling age are more susceptible than adult ones. Recommended ages for different species are: guinea pig - 1 week (120-140 grams); hamster - 21 days (18-25 grams); deermice, rabbits and gerbils - 10 days; chicks - 1-3 days; and Swiss mice weighing less than 10 grams. White mice were not recommended for the isolation of leptospires as, frequently, the mice colonies

were found to be naturally infected with various serotypes of leptospires.

Alexander et al. (1970) reported that while guinea pigs and hamsters were the animals of choice, young animals regardless of species were more susceptible than older animals. All laboratory animals inoculated were held for 10 days before they were sacrificed and tissues collected.

The advantages of animal inoculation include the following: Material unsuitable for culture can be used for animal inoculation. A fairly large quantity (1-5 ml) can be inoculated in animals. Leptospires multiply rapidly in a susceptible living host and can be demonstrated within a few days. Disadvantages include the cost of maintenance of laboratory animals, and mice colonies may be found to be naturally infected with L. ballum.

C. Serological methods.

Several serological methods are available for the diagnosis of leptospirosis. Antibodies generally appear following the 6th day to 12th day of the illness. The antibody titer may reach maximum by the 3rd or 4th week. Low titers may persist for a long time after the infection has subsided. It is therefore not possible to examine a single serum sample and say whether the infection is recent or a residual effect of a previous infection. It is imperative to test at least two serum samples, one taken during the early course of a disease and a second taken a week or two later. A significant rise in titer (4 fold) is considered as an indication of current infection. Serological methods do not provide early diagnosis but they are of value in confirming an earlier diagnosis. There are several factors, such as the time of collection of blood samples during the course of the disease, the type of test applied, antibiotic therapy and handling of specimen, which influence the interpretation of the results (Galton et al., 1962; Turner, 1968).

1. <u>Agglutination tests.</u>

a. Microscopic agglutination test. Microscopic agglutination test originally described by Schuffner and Mochter (1927) is conducted with live leptospires as antigens. The test is highly sensitive and specific and can be applied to human as well as animal sera.

Test procedure: 5-7 day old living culture of a strain of a specific leptospiral serotype is used as the antigen. Serial 4-fold dilutions of the suspect serum are made in phosphate buffered saline, pH 7.4, ranging from 1:50 to 1:12,800. For each dilution 0.2 ml is mixed with 0.2 ml of suitably diluted antigen and incubated at $30^{o}C$ for 3 hours. A small drop from each tube is examined under darkfield with low power (100X) without coverslip. The reaction is graded as negative, trace, partial, and complete depending on the degree of clumping of leptospires. The end point is a dilution at which 50% of leptospires are agglutinated. A less sensitive test can be done utilizing formalin killed leptospires as the antigen (Wolff, 1954).

Stoenner (1955) studied the effect of density of antigen, method of preparing dilutions, strain of leptospires, incubation period, and age of culture on the results of agglutination tests. He observed that density of antigen and method of preparing the dilution influenced the titers more than the strain of leptospires, incubation time or age of culture. Antigens with low cell counts and 10-fold serum dilutions gave higher titers than with dense antigens and 2-fold serum dilution.

The World Health Organization (Anon., 1967b) expert group in their report considered the microscopic agglutination test as the standard test for the serological diagnosis of leptospirosis. They standardized two variables, the antigen density and definition of end point, that might influence the

interpretation of results. They recommended 4-14 day old cultures with a density of 100 million leptospires per ml in the final serum antigen mixture. They defined the end point as the highest final dilution of serum in the serum antigen mixture in which 50% or more of the leptospires are agglutinated.

Turner (1968) reported that the word "lysis" formerly used in the agglutination test is no longer applicable as it has been shown that the test does not result in lysis of the organisms as formerly believed. The test is now called the microscopic agglutination test with live antigen (MAL) and microscopic agglutination test with killed antigen (MAK). Caccihone et al. (1969) modified the microscopic agglutination test by maintaining the tubes at 45°C for 20 minutes in the water bath instead of 2 hours in incubator at 37°C. The results of the modified method compared favorably with the conventional method when tested on 326 serum samples.

Ryu (1970) conducted the microscopic agglutination test with a drop of blood absorbed on a filter paper of 1 cm x 5 cm, dried and stored at -20°C. The filter paper was placed in a tube containing 1 ml phosphate buffered saline. When the serum was dissolved in buffer 1 drop of diluted serum and 1 drop of antigen were mixed and kept at room temperature for 5 minutes, and examined under darkfield. The reaction was considered as positive when at least one swollen leptospirum or one microbial clumping was present in each darkfield.

b. Macroscopic tube agglutination test. Howarth (1956) described the macroscopic tube agglutination test in which formalin killed culture was used as antigen.

c. Macroscopic plate agglutination test. Galton et al. (1958) described a macroscopic plate agglutination test. Formalin killed cultures of

leptospires suspended in 12% sodium chloride and 20% glycerine was used as antigen. When compared with microscopic agglutination test (MAL) a close correlation was observed. Tests with pooled antigens were found to be suitable for screening work.

Crawford (1964) compared macroscopic plate agglutination tests with microscopic agglutination tests on 548 bovine serum samples. The results compared favorably and he reported that macroscopic plate agglutination test was a valuable tool in screening.

Solorzano (1967) compared Galton's macroscopic slide agglutination test with the microscopic agglutination test. He reported that some formalinized antigens formed coarse clumping with normal saline and negative control serum. He failed to get the high correlation between the two tests.

Lepherd (1969) reported that macroscopic plate agglutination test was not suitable to accurately detect leptospiral antibodies in horse sera.

Chernesky (1970) applied macroscopic slide agglutination and microscopic agglutination tests for the survey of canine leptospirosis in British Columbia. He reported that macroscopic test was suitable for screening but the microscopic test was superior in detecting the low titers.

d. Micro-agglutination test. Galton et al. (1965) described the micro-agglutination test. The test was conducted in a plastic plate with U wells. The serum was diluted from 1:25 to 1:12,800 in a final volume of 0.025 ml. The reaction was read on the plate itself under a zoom disecting microscope and graded from negative to 4+. With this method they claimed saving of 75 to 80% of time and 80-fold serum and antigen.

Fuchs (1969) reported that the titer in the micro-agglutination test was influenced by the age of the culture, and the concentration of leptospires.

e. Sensitized erythrocyte agglutination (SEA) and sensitized erythrocyte lysis (SEL) tests. Chang and McComb (1954) observed an erythrocyte sensitizing substance (ESS) in alcoholic extracts of leptospires. They described SEA and SEL tests for the diagnosis of leptospirosis. The tests were found to be specific on human sera. They compared the SEA test with the SEL test (Chang et al., 1957) and found that SEL test was more sensitive and of diagnostic value.

McComb et al. (1957) and Sharp (1958) evaluated the tests and found them to be in agreement with microscopic agglutination test. SEL titers were observed to appear and disappear earlier than agglutination titers. They reported that SEL was of diagnostic value because of its broad specificity and ability to detect recent infection. Gochenour et al. (1958) reported that SEA test was not suitable for animal sera. Meers and Ringrose (1968) simplified the SEL test by using multiple depression trays, automatic syringes, and commercially available buffer. The test was found to be genus specific and sensitive but of limited value in detecting past infection.

f. Hemagglutination (HA) and hemolytic (HL) tests. Cox (1955) described the hemagglutination and hemolytic tests for diagnosis of leptospirosis. One volume of a 10% suspension of washed sheep erythrocytes was mixed with 10 volumes of leptospiral extract, incubated in water bath at $37^{o}C$ for 1 hour. Final suspension of 1% sensitized cells was made.

HA procedure: 0.1 ml of sensitized erythrocytes was mixed with 0.4 ml of serum dilutions, incubated at $30^{o}C$ for 16-20 hours and the results read.

HL procedure: 0.1 ml of sensitized erythrocytes was mixed with 0.4 ml of inactivated serum dilution. Two units of guinea pig complement in 0.2 ml were added to each tube, incubated for 1 hour at $37^{o}C$ in water bath. The

results were graded as complete, partial and negative. They observed that HL test was extremely group specific. The HA test was similar in pattern to the HL test, but the titers were lower. They recommended the HL test for screening.

Cox et al. (1957) conducted HL test by using extracts from L. biflexa. When the test was evaluated on 181 serum samples from known positive cases 179 samples gave high titer. The HL titers were observed to appear early in the course of the disease. They recommended the use of extracts of L. biflexa as sensitizing substance because of its broad specificity. Gochenour et al. (1958) reported that HA and HL tests were not suitable for diagnosis of human leptospirosis.

Stauch and Hopps (1968) described hemagglutination tests with formalinized sheep erythrocytes acting as a carrier for the hemolytic antigen derived by alcoholic fractionation of heat killed L. biflexa. In a positive reaction the cells settled to form a mat and in negative sera the cells formed a button.

g. Latex agglutination test. Muraschi (1958, 1959) described the latex agglutination test for the diagnosis of leptospirosis. The antigen was prepared by adsorbing formalin killed leptospires to polysterene latex particles. The test compared favorably with the microscopic agglutination test. Pooled antigens were useful for screening.

Kelen and Labzoffsky (1960) modified the latex agglutination test by adsorbing soluble antigen to latex particles.

2. Complement fixation test (CFT). Randall et al. (1949) described the CFT with sonic vibrated leptospires as the antigen. The antigen was stable

for at least 6 months. The test was reported to be 3 times more sensitive with sonic vibrated antigen than with live antigen.

York (1952) described CFT using leptospires grown in chick embryo as antigen. The test was reported to be specific and sensitive. Pike et al. (1954) described the CFT done on a plate. The advantages claimed were a relatively small amount of antigen, economy in other materials and glassware. They reported however that further trials were necessary to evaluate the test as a diagnostic tool.

Muraschi et al. (1956) described the CFT with ethylene glycol extracts of leptospires as antigen. CFT titers were detected early in the disease. Pooled antigens were equally good when used for screening.

Schubert et al. (1956) used whole leptospires as antigen in CFT. It was reported to be superior to supernate antigen and sonic vibrated antigen. They observed that supernate antigen failed to fix complement with sera from infected human cases and cattle.

Rothstein and Wolman (1959) used ethanol extracts of leptospires as antigens in CFT. The antigen was stable for 4 years at 4°C. They reported that CFT titers could be detected as early as 2-3 days from the onset of the disease.

Sturdza et al. (1960) used an antigen prepared from L. biflexa (Patoc. strain) in CFT. They observed that the antigen was good for the detection of antibodies against 8 serotypes most commonly found in Roumania. The CFT compared favorably with microscopic agglutination test on 36 human sera. They concluded that the use of this single antigen eliminated the need to maintain several cultures. The CFT titers were detected earlier than the agglutination titers. The test was reported to be suitable to detect recent infection.

Marius and Nicora (1964) used L. biflexa (Patoc. strain) as antigen in the CFT. They reported close correlation between CFT and agglutination tests. The test was found to be genus specific. In view of its broad specificity they recommended CFT with L. biflexa (Patoc. strain) as antigen for screening.

D. Fluorescent antibody technique (FAT).

Coons et al. (1941) demonstrated that antibody could be labeled with fluorescein dyes without impairing the properties of the antibody. Coons et al. (1942) applied FAT for the first time in the field of diagnostic microbiology. They demonstrated pneumococcal antigen in the tissue sections of infected mice. Coons and Kaplan (1950) modified the synthesis of isocyanate and developed isomer II of nitro fluorescein isocyanate (FIC). They also introduced adsorption of the conjugate with mouse liver powder as a means of reducing nonspecific staining.

Riggs (1954) synthesized fluorescein isothiocyanate (FITC) which was found to be superior to all fluorescein dyes available to conjugate the serum. Marshall et al. (1958) compared FIC and FITC and reported that the FITC was superior.

The advantages of FAT are two fold. Leptospires are stained so they can be seen under the fluorescent microscope and it is a specific antigen-antibody reaction. A second advantage of the FAT is that the antigen can be detected in a material which is otherwise unsuitable for culture due to bacterial contaminants. FAT is effective even with a ratio of contaminants to specific cells as high as 10^7 (Cherry et al., 1965; Turner, 1970).

1. <u>Staining methods</u> (Cherry <u>et al</u>., 1965; Goldman, 1968; Nairn, 1969; Kawamura, 1969).

a. Direct. In this method labeled antibody is directly applied to the smears or tissue sections. The method is simple and specific. The disadvantage is that antibody against each antigen has to be labeled. This technique is preferred for delicate examinations due to its high specificity.

b. Indirect. In this method the antigen is first exposed to unlabeled antibody followed by exposure to a conjugated antibody directed against the unlabeled antibody. The advantage of this method is that a single labeled antibody can be used to stain any antigen provided the non-conjugated specific antibody is obtained from an animal against which the conjugated anti-gamma globulin is available. This method is considered to be more sensitive than the direct method as more sites on the previously adsorbed antigen are available for staining.

The procedure in general is the same as in direct, except the slide is treated first with unlabeled specific antibody and then with labeled antibody directed against the unlabeled, specific antibody instead of against the antigen.

c. Complement. This is a modification of the indirect method. This method is used where antigen-antibody complexes combine with the complement. Specific antibody directed against complement is labeled. In this method inactivated antibody, fresh guinea pig complement and labeled antibody against guinea pig complement are used. The advantage of this method is that one labeled antibody directed against guinea pig complement can be used to stain any antigen antibody complexes which combine with complement regardless of the source of antibody.

2. <u>Non-specific staining</u>. In all the methods described one of the major problems acknowledged was non-specific staining. Coons et al. (1950) adsorbed conjugate with mouse liver tissue powder to reduce the non-specific staining. Curtain (1961) observed that non-specific fluorescence was due to soluble fluorescein derivatives. He purified the conjugate by filtering it through a Sephadex column. Goldstein <u>et al</u>. (1961) reported that exclusion of serum proteins other than gamma globulin or the unreacted fluorescein isothiocyanate did not diminish the non-specific fluorescence. They reported that optimum fluorescein to protein ratio (F:P) was significant.

3. <u>Application of FAT in the diagnosis of leptospirosis</u>. Sheldon (1953) demonstrated leptospires by FAT in a muscle biopsy from a human patient. Warthin-Starry staining of sections failed to reveal leptospires.

Moulton and Howarth (1957) demonstrated <u>L</u>. <u>canicola</u> in experimentally infected hamster kidneys by FAT. They also demonstrated leptospires by FAT on smears made from Millipore filter membranes through which the broth culture of isolated leptospires was passed. They observed that staining by FAT of urine smears was unsatisfactory due to the brilliant non-specific background fluorescence and leptospires being disintegrated by centrifugation. They concluded that staining by FAT of smears was less satisfactory because of the difficulty in obtaining leptospires in sufficient concentration.

White and Ristic (1959) demonstrated <u>L</u>. <u>pomona</u> in urine of experimentally infected guinea pigs and calves by FAT where darkfield examination failed. They recommended FAT for detecting carrier shedders. Maestrone (1961) found that it was possible to demonstrate leptospires in embryonated eggs by FAT from the start of the infection to study its pathogenesis. White <u>et al</u>. (1961) demonstrated leptospires by FAT in urine preserved in formalinized

phosphate buffered saline (PBS). They reported that when the leptospires were few in number bladder tapping and culture was preferable to FAT.

Boulanger and Robertson (1961) applied FAT on smears of pure culture of L. pomona, urine, and kidney impressions. They reported that dry films fixed in formalin were not satisfactory. They preferred absolute alcohol as a fixative. In swine and calf urine FAT were positive when darkfield examinations were positive. In kidney impression smears leptospires were observed by FAT as well as by darkfield examination. Where 4 kidney cultures were positive only 2 were positive by FAT. They concluded from their studies that FAT was less effective with fewer leptospires in urine, lower in efficiency than culture, nearly as effective as darkfield examination.

Dacres (1961, 1963) reported that it was possible to identify the leptospires serotype by cross staining provided the tissue section was frozen and fixed with osmic acid vapor.

Coffin and Maestrone (1962) compared FAT with darkfield microscopy and cultural methods for examination of fresh and preserved specimens. Culture smears were fluorescent after storage for one year at room temperature without fixing. In dog's urine darkfield micropsy revealed leptospires for a few hours, but could be detected by FAT following storage at room temperature for 20 days, for 9 months at 4°C, for 9 months at room temperature with addition of formalin 0.5 to 2%. In 20 samples of urine from infected dogs leptospires were demonstrated in all 10 by FAT but in only one by darkfield examination. They found that the staining properties of leptospires in dired smears from urinary sediment remained unchanged for at least 6 months when stored at 4°C. Leptospires were detected by FAT but not by darkfield examination or culture under the following conditions: Allantoic fluid with pH 5; storage of tissue suspensions at room temperature for a week; presence of contamination; storage

at 4°C for 3 days; storage at -30°C for 20 minutes; and freezing and thawing

10 times. They reported that the effect of formalin on leptospires could be

reversed by treating slides with ammonium hydroxide and leptospires were

demonstratable in tissues fixed in formalin up to 18 months. They concluded

that FAT is specific, useful in detection of a minimal number of leptospires

even when dead, and rapid in diagnosis.

Maestrone (1963) described a modification of FAT for staining formalin

fixed tissues. He observed that application of Tween 80 (3%), a surfactant,

before the application of the conjugate improved the brightness four fold. He

applied FAT for the detection of leptospires in formalin fixed tissues from

dogs, guinea pigs, hamsters, cattle, pigs, certain wild animals and equine,

bovine and swine fetuses. He stated that FAT was superior to culture and to

animal inoculation.

Kellogg and Deacon (1964) described a new rapid FAT for the demonstration

of Treponema pallidum in human syphilitic lesions. Impression smears from

human syphilitic lesions were air dried and fixed by heat. A 6 mm diameter

circle was marked on the fixed slide with a diamond pencil and covered with

conjugate. The conjugate was allowed to dry on the smear at 45°C. The slide

was rinsed under running tap water, buffered saline, or distilled water for 5

seconds and mounted under glycerol buffered saline. The test was compared with

the conventional method. The brightness was essentially the same as with con-

ventional method. The rapid method was specific. It did not stain other

bacteria including other spirochetes, yeast or fungi. The advantages claimed

were rapidity (the entire process took not more than 5 minutes), simplicity,

and specificity.

Radu et al. (1965b) compared FAT with darkfield examination. Serial

dilutions of cultures of L. icterohaemorrhagiae were tested for the presence

of leptospires by FAT and darkfield examination. FAT proved to be 8 times more sensitive than darkfield examination. They were able to detect leptospires by FAT up to the dilution of 1:320,000. In experimentally infected guinea pigs they were able to detect leptospires in blood smears by FAT, whereas darkfield examination, Giemsa stains and culture techniques failed. They also compared FAT with agglutination lysis test (1965a) and found a close correlation between the two. However they observed more cross reactions to occur in FAT than in agglutination lysis test.

Schröder (1966) demonstrated leptospires in two newborn piglets by FAT which were negative for culture.

Duplesis (1966) compared indirect fluorescent antibody techniques (IFAT) with the microscopic agglutination test for the diagnosis of leptospirosis. He found that IFAT was group specific whereas agglutination test was serotype specific. However he recommended the IFAT for screening serum samples for the detection of antibodies against leptospires because of its group specificity, availability of commercially prepared labeled antibody. He stored antigen smears at -20°C for 6 months, and found that stained slides could be examined after several days of storage at 4°C.

Horsch et al. (1966) demonstrated leptospires in liver and kidney impression smears from two piglets by FAT but attempts to culture failed. L. pomona was isolated from urine of the sow.

Torten et al. (1966) used L. biflexa (Strain Patoc I) as the antigen for IFAT and compared with agglutination test. They reported from their studies on 120 serum samples from suspected human cases that IFAT with L. biflexa (Strain Patoc I) was specific and rapid. The advantages claimed were that positive IFAT results indicated recent infection as IFAT titers were found to disappear earlier than agglutination titers.

Smith et al. (1967) demonstrated leptospires in autolytic ovine and bovine fetuses by FAT but attempts to culture leptospires failed. Fragments of leptospires were not detectable by silver staining but it was possible to detect them by FAT.

Rosie et al. (1967) subjected impression smears from 241 human syphilitic lesions to FAT and darkfield examination. They reported that results with FAT were better than with darkfield. Atypical lesions were positive only by FAT. They recommended FAT for the diagnosis of syphilis as it was reliable and rapid and especially useful where the facilities for examination were not available. In the latter case smears were sent to a laboratory for diagnosis.

Schröder and Senf (1967) demonstrated L. hyos in kidneys of aborted swine fetuses and in urine sediment of the sow by FAT.

Sulzer et al. (1968) reported a survey on 200 rats trapped in the metropolitan area of Atlanta, Georgia. Sera from 136 rats were examined by IFAT, culture techniques and the slide agglutination test. They found a better correlation between slide agglutination and culture than IFAT with culture. However where serum samples were positive by only one of the three methods, the highest percentage was found by IFAT (10% by culture, 18% by IFAT, 3.5% by slide agglutination test).

Hirschberg et al. (1968) applied IFAT on human serum samples obtained from normal individuals, patients proved or suspected of having leptospirosis, and patients positive for diseases other than leptospirosis. Thirty-two samples which were negative by the slide agglutination test were positive by IFAT. In 13 paired serum samples from acute and convalescent patients 92% were positive by IFAT but only 46% by the agglutination lysis test. All convalescent serum samples were positive by both methods. In 32 samples from

normal individuals 2 were positive by IFAT but all were negative by agglutination lysis test. They commented that the IFAT test was more efficient than conventional methods in that IFAT titers could be detected earlier than agglutinating titers.

Burger and Fuchs (1968) reported on 39 serum samples from cattle that IFAT was more sensitive than the microagglutination test.

LeClair (1969) evaluated the IFAT with L. biflexa (Strain Patoc) as the antigen for the serodiagnosis of leptospirosis. The factors considered for the evaluation were sensitivity, specificity, reproducibility and percentage of agreement with the agglutination lysis test. The test was evaluated on serums from experimental guinea pigs infected with pathogenic leptospires, from cattle clinically diagnosed as having leptospirosis, and from human cases of leptospirosis. They observed that sensitivity of IFAT was greater than that of agglutination lysis test during the first week of infection in guinea pigs. The specificity was found to be greater than 95% in all cases except on sera from cattle in which it was unsatisfactory. They reported that IFAT was of value in differentiating leptospiral serotypes when antiserums were diluted to their homologous titer to avoid cross reactions.

Sturdza (1969) compared IFAT with darkfield examinations of kidney impression smears from 100 sows slaughtered from farms known to be infected with leptospirosis. Leptospires were demonstrated in 29 smears by darkfield examination and by IFAT in 36 smears.

Cook (1970) reported that leptospires could be demonstrated by direct FAT in sections of tissues preserved in 10% phosphate buffered neutral formalin up to 464 days without loss in staining intensity or specificity, and up to 618 days by IFAT.

All diagnostic methods described above have certain limitations. However FAT for the demonstration of leptospires in fluids and tissues offers a promising method for the diagnosis of leptospirosis (Anon., 1967b).

MATERIALS AND METHODS

Leptospira Serotype and Strain

Serotype <u>canicola</u> (Strain Moulton Dog Clone 36 HP) and serotype <u>canicola</u> (Strain Sow 152) were obtained from the World Health Organization Leptospirosis Reference Laboratory, Division of Veterinary Medicine, Walter Reed Army Institute of Research, Washington, D. C. In this study serotype <u>canicola</u> (Strain Moulton Dog Clone 36HP) was used. Cultures for routine work were maintained in Stuart's medium[a] and transferred at 5-7 day intervals. Stock cultures were maintained in Fletcher's medium.[a] After multiple transfers in Stuart's medium 4-5 passages were made in 17-day-old hamsters to enhance virulence. Leptospires recovered from hamsters were maintained in Fletcher's medium as stock cultures. Leptospires counts were made with a Petroff-Hausser bacteria counter.[b]

Antileptospiral Serum

Antileptospiral serum was prepared in rabbits in accordance with the procedures employed by the Walter Reed Army Institute of Research (Alexander, 1958). Normal adult rabbits were inoculated intravenously at 7 day intervals with successively increasing doses of 1.0 ml, 2.0 ml, 4.0 ml and 6.0 ml of 5-day-old live leptospires cultures grown in Stuart's medium. The rabbits were exsanguinated on day 6 following the last inoculation. The serum was

[a]Difco Laboratories, Detroit, Michigan.

[b]Arthur H. Thomas Co., Philadelphia, Pennsylvania.

collected and filtered through a Seitz filter or a Nalgene 0.20 u plain membrane filter unit[c] and stored in 5 or 10 ml quantities in sterile, screw-capped pyrex test tubes at -20°C. The antibody titer was determined by plate agglutination tests, and microscopic agglutination tests. The titer was 1:12,500 by the microscopic agglutination test and 1:320 complete and 1:640 incomplete by the plate agglutination test.

Conjugation of Antibodies with Fluorescein Isothiocyanate (FITC) (Goldman, 1968; Kawamura, 1969; Nairn, 1969)

A. Preparation of serum globulins.

The serum was initially diluted 2-fold with 0.1 M phosphate buffered saline (PBS), pH 7.2. An equal volume of saturated ammonium sulfate (pH adjusted to 7.0 and filtered) was added drop by drop by using a burette to bring to 50% concentration. During the process the tube contents were stirred constantly by a magnetic stirrer and the tube was kept in an ice bath. The mixture was then allowed to stand for 30 minutes. It was then centrifuged in a refrigerated centrifuge[d] at 12,062 g (10,000 RPM) for 10 minutes. The supernatant was then measured and discarded. The precipitate was resuspended by separately adding equal volumes of PBS (first) and saturated ammonium sulfate to equal the original volume of supernatant discarded. Centrifugation was repeated. The precipitate resuspended and the entire process repeated. The final precipitate was resuspended in small amounts of PBS (approximately 2 ml) and dialyzed against PBS in a refrigerator until all the NH_4 or SO_4 ions were removed. The PBS was changed 3-4 times during the process. To assure

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[c]NALGE Sybron Corporation, Rochester, N. Y.

[d]Sorvall RC2-B, New Town, Conn.

that the outer fluid was free from sulfate ions, 150 mg of barium chloride were dissolved in 3 ml of distilled water and mixed with 3 ml of the PBS from the outer container. The PO_4 ions from the PBS formed a white precipitate. The precipitate disappeared upon the addition of a few drops of dilute hydrochloric acid. If the PBS was not free of NH_4 and SO_4 ions a significant amount of precipitate remained following the addition of hydrochloric acid. Following dialysis the protein concentration was determined by refractometer.[e]

B. Labeling the antibody.

The pH of the dialyzed globulin solution was adjusted to 9.5 using a 0.5 M carbonate bicarbonate buffer pH 9.5. An equal volume of 0.5 M carbonate bicarbonate buffer was added to the dialyzed globulin solution, saving 1 ml to dissolve FITC.[f] FITC equal to 1/150 of the total protein was dissolved in 1 ml of 0.5 M carbonate bicarbonate buffer pH 9.5, and 3-4 drops were slowly added every 10 minutes to the buffered globulin solution. This process was done under refrigeration and a magnetic stirrer was used to stir the solution for 4 hours to allow adequate conjugation to take place.

C. Removal of the free dye.

Unconjugated dye was removed by filtering the conjugate through a Sephadex column. A chromotography column prepared with Sephadex G-25 (fine)[g] was equilibrated with 0.005 M PBS pH 7.0. The conjugated globulin was applied to the column and eluted with 0.005 M PBS. Eluted fractions containing the

[e]TS Meter. American Optical Corporation, Buffalo, N. Y. 14215.

[f]Nutritional Biochemical Corp., Cleveland, Ohio 44128.

[g]Pharmacia Fine Chemicals, Inc., Piscatway, N. J.

purified conjugate (examined with U.V. light[h]) were collected in 1-2 ml amounts and stored at -20°C.

Adsorption of Conjugated Antibodies

A. Preparation of tissue powders.

Acetone dried dog liver and testis tissue powders were used for adsorption of the conjugate.

Procedure: 20 Gm of fresh testis (or liver) was obtained from a dog, free from serum leptospiral antibodies, after exsanguination. The tissue was cut into small pieces and washed with distilled water several times and homogenized in 20 ml of normal saline under refrigeration. One hundred-sixty ml of acetone were added to the homogenate while stirring. The homogenate was centrifuged at 1,085 g (3,000 RPM) for 10 minutes in refrigerated centrifuge (Sorvall RC-2B). The supernatant was discarded and the sediment was resuspended in 80 ml of saline and refrigerated overnight. The suspension was again centrifuged and the sediment resuspended in 20 ml of saline. Resuspension in saline and treatment with 160 ml aliquots of acetone were repeated until hemoglobin pigments were no longer visible in the supernatant. The acetone treated sediment was resuspended in 80 ml of acetone and was allowed to stand for 30 minutes, stirred, and the supernatant removed. This was repeated twice. The final sediment was spread on filter paper and dried at 37°C in an incubator. The dried tissue powder was bottled and stored at 4°C.

B. Adsorption techniques.

Fifty mg of tissue powder was added per ml of conjugate for the initial adsorption, and 25 mg/ml for the second.

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[h]Black Ray - UVL, Ultra Violet Products, Inc., San Gabriel, California.

Procedure: The tissue powder was presoaked in PBS for one hour and centrifuged at 12,062 g (10,000 RPM) for 20 minutes. The supernatant was discarded and the conjugate added to the sediment. The mixture was allowed to stand for one hour, with frequent stirring. It was centrifuged at 30,900 g (16,000 RPM) (Sorvall RC-2B) for 20 minutes. The supernatant was carefully collected. A second batch of tissue powder (presoaked in PBS and centrifuged) was added to the supernatant, mixed thoroughly and allowed to stand for one hour, with frequent stirring. The mixture was centrifuged at 30,900 g (16,000 RPM) for 20 minutes. The adsorbed supernatant was collected and dispensed in 0.5 ml quantities into bottles and stored at -20°C.

C. Titration of the adsorbed conjugate.

To determine the optimum dilution of the conjugate, smears made from urine containing leptospires (culture added) and formalinized to 0.8% final concentration were stained with conjugate (direct FAT) diluted 1:4, 1:8, 1:16, 1:32 and 1:64 in PBS. The optimum dilution was determined on the basis of maximum fluorescence intensity with minimum background fluorescence.

Collection and Processing of Materials

A. Survey of dogs for the detection of shedders.

1. Collection of urine. Urine specimens from dogs brought to Dykstra Veterinary Hospital were collected when they were exercised. Midstream urine specimens were collected. Immediately after collection one drop of urine was inoculated into a tube containing 5 ml Stuart's medium (Menges et al., 1960). The urine pH was adjusted to 7.2 - 7.4 using 2 M sodium hydroxide (Alexander et al., 1970) and 1 ml of PBS pH 7.6 was added to each 10 ml of urine (White and Ristic, 1959). In the laboratory 15 ml of urine was centrifuged at 381 g

(1,500 RPM) for 15 minutes to remove the gross particles. Two ml of the supernatant were passed through a cellulose filter,[i] 0.45 u pore size, 13 mm diameter, using a Swinny filter holder. The initial 3-4 drops were discarded and the next 4-6 drops were inoculated directly into the Stuart's medium. The tubes were incubated at 30°C and examined by darkfield microscope after 7 days. If the tubes were negative for growth they were again examined at the end of the 4th week before they were discarded. The remaining supernatant was formalinized to 0.8% of final concentration and preserved for further examination.

2. Preparation of samples. (a) Millipore filter membrane: Clean glass microscope slides were dipped in 1% gelatin solution and allowed to dry. The filter membrane was removed from the Swinny filter holder and placed with the filtering side against the microscope slide. Applying gentle pressure, a smear was made on the slide with a single forward stroke. The smear was then allowed to air dry before processing.

(b) Formalinized urine: Formalinized urine (10 ml) was centrifuged at 1,522 g (3,000 RPM) for 45 minutes in Sorvall G-L-C-1 centrifuge. The supernatant was pipetted and the sediment removed. A drop of the sediment was placed on a gelatin treated slide and spread on the slide with gentle firm forward pressure using a Number 11 Bard Parker blade. The smear was then air dried before processing.

3. Staining. The air dried smears were fixed in acetone for 5 minutes and dried for 5 minutes at 37°C. Two 1.2 cm circles were etched on the slide with a diamond point pencil and marked with a Mark Tex-Tech Pen.[j] A drop of previously diluted conjugate was placed in each inscribed circle and spread to

[i]Millipore Corporation, Bedford, Mass. 01730.

[j]Mark-Tex Corporation, Englewood, N. J.

cover the entire area. The slides were incubated in moist chambers (petri dishes containing moistened filter paper) at 37°C for 30 minutes. The excess conjugate was drained off and the slides washed two times, 5 minutes each, in PBS. They were then blotted dry and counter-stained with Lissamine Rhodamine FA counterstain[k] (diluted 1:20 as per the instructions) for 30 seconds, washed two times in PBS, 5 minutes each. The specimens were blotted dry, covered with pH 7.2 buffered glycerine and coverslipped and immediately examined.

B. Experimental studies in dogs.

Four dogs, 6 months to 1 year old, of mixed breeds, free from shed leptospires (by FAT on urine) and serum leptospiral antibodies (by the plate method) were used in the experimental studies. The dogs were inoculated intraperitoneally for 4 days successively with 4.5 ml of a 5-day-old culture (containing 1.8×10^9 to 2×10^8 leptospires per ml) of L. canicola (Strain Moulton Dog Clone 36HP) grown in Stuart's medium. Morning and evening rectal temperatures were recorded daily. At the peak of body temperature response, the blood was examined under darkfield and Stuart's and Fletcher's media tubes were inoculated (irrespective of the results of darkfield examination). Blood was collected at the end of the 1st week for serology. From the beginning of the 2nd week freshly voided urine was collected and examined by darkfield and FAT. When leptospires were detected by either method, voided urine and bladder tap specimens were collected and cultured (Menges et al., 1958, 1960; White and Ristic, 1959).

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[k]Difco Laboratories, Detroit, Michigan.

C. Experimental isolation of leptospires from urine containing known concentrations of leptospires.

1. Urine culture. Fresh voided urine from dogs free from leptospiral serum antibodies (by plate test) and shed leptospires (by FAT on urine) was collected and about 10 ml were centrifuged at 381 g (1,500 RPM) in Sorvall G-L-C-1 centrifuge for 15 minutes. Three ml of supernatant were first passed through Millipore filters to remove the contaminants (Rittenberg et al., 1958). Bacterial counts were made on 5-7 day old leptospires cultures grown in Stuart's media. Serial dilutions of the cultures were made in fresh Stuart's media and the final dilution in filtered urine. Known number of leptospires were inoculated into 3 tubes of Stuart's medium. All trials were duplicated twice. The tubes were incubated at 30°C for 5 days and examined under dark-field. If the first examination was negative for growth, the tubes were again examined at weekly intervals for 3 additional weeks before they were discarded as negative. The trials were conducted to a dilution end point where no growth was observed in any of the tubes inoculated.

2. Darkfield microscopy. A 5-7 day old culture with known leptospires counts was serially diluted in fresh urine. A drop from each dilution was examined under darkfield for the detection of leptospires. If the result was negative with one examination, 3 additional slides with 2 drops on each were examined, both under 100X and 400X magnification, before the trial was considered negative.

3. FAT. Five ml of each dilution were centrifuged at 381 g (1,500 RPM) in Sorvall G-L-C-1 centrifuge for 45 minutes. Sediment smears were stained by the direct FAT and examined for leptospires.

D. Fluorescent microscopy.

A Leitz Ortholux research microscope[1] equipped for transmitted light fluorescence with 4 mm BG 38 heat absorbing filter, 3 mm BG 12 blue excitation filter, K510, K530 barrier filters, and an immersion darkfield condensor D 1.20 was utilized. An Osram mercury vapor lamp[m] HBO 200W L-2 was used as the source of light. For routine examination of FA slides 10X eye pieces and 25X and 40X dry objectives were used; for detailed studies and photography a 54X oil immersion objective was used. A Leitz Orthomat 35 mm automatic microscope camera and Ektochrome high speed daylight film ASA 160/Din 23 were used for photography.

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[1] E. Leitz Inc., 468 Park Avenue South, New York, N. Y. 10016.

[m] E. Leitz Inc., Rockleigh, N. J. 07647.

RESULTS

TABLE I. Results of Survey of Canine Urine Samples for Leptospires Shedders by Fluorescent Antibody and Cultural Techniques.

Total number examined	FAT on Millipore filter membrane impression smears	FAT on formalinized sample smears	Urine culture[*]
591	19 pos.	neg.	neg.

[*]Stuart's medium.

Pos. = Positive for leptospires.

Neg. = Negative for leptospires.

TABLE II. Results of Blood Examination by Darkfield and Culture on Dogs Experimentally Infected With _Leptospira canicola_ (Strain Moulton Dog Clone 36HP).

Dog #	96			97			98			99		
P.I. Day	Body temp. C°	Dark-field	Culture*	Body temp. C°	Dark-field	Culture*	Body temp. C°	Dark-field	Culture*	Body temp. C°	Dark-field	Culture*
2	NE	NE	NE	A.M. 39.4	Neg.	Pos.	NE	NE	NE	NE	NE	NE
				P.M. 41.6	Pos.	Pos.						
3	39.3	Neg.	Pos.	40.0	Neg.	Pos.	41.0	Neg.	Pos.	39.8	Pos.	Pos.
4	39.8	Neg.	Pos.	38.6	NE	NE	39.2	Neg.	Pos.	39.3	Neg.	Pos.
5	39.2	NE	Pos.				39.0	NE	NE	39.6	NE	Pos.
6	38.6	NE	Pos.							39.5	NE	Pos.
7	38.6	NE	NE							38.4	NE	NE

*Stuart's and Fletcher's media.

P.I. Day = Post inoculation day.   NE = Not examined.   Neg. = Negative for leptospires.

Pos. = Positive for leptospires.

TABLE III. Results of Urine Examination by Darkfield, Fluorescent Antibody Techniques (FAT) and Culture on Dogs Experimentally Infected with _Leptospira canicola_ (Strain Moulton Dog Clone 36HP).

Post inocula-tion day	96			97			98			99		
	Dark-field	FAT	Culture*	Dark-field	FAT	Culture*	Dark-field	FAT	Culture*	Dark-field	FAT	Culture*
9	NE	NE	NE	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.
13	Neg.	Neg.	Pos.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	Pos.
14	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.
15	Neg.	Neg.	Neg.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	Neg.
16	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	NE
17	Neg.	Neg.	Neg.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	NE
18	NE	NE	NE	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	NE	NE	NE
19	NE	NE	NE	NE	NE	NE	Neg.	Neg.	NE	NE	NE	NE
20	Neg.	Neg.	Neg.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	Neg.
21	Neg.	Neg.	Neg.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	NE
22	Neg.	Neg.	Neg.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	NE
23	Neg.	Neg.	Neg.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	NE
24	NE	NE	NE	NE	NE	NE	Neg.	Neg.	Neg.	NE	NE	NE
27	Neg.	Pos.	Neg.	NE	NE	NE	NE	NE	NE	Neg.	Neg.	NE

TABLE III (Continued)

Dog #	96			97			98			99		
Post inoculation day	Dark-field	FAT	Culture*	Dark-field	FAT	Culture*	Dark-field	FAT	Culture*	Dark-field	FAT	Culture*
28	NE	NE	NE	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	NE	NE	NE
29	Neg.	Pos.	Neg.	Pos.	Pos.	Neg.	NE	NE	NE	Pos.	Pos.	Pos.
30	NE	NE	NE	Pos.	Pos.	Pos.	Neg.	Neg.	Neg.	NE	NE	NE
31	Neg.	Neg.	Neg.							Pos.	Pos.	Neg.
37	Pos.	Pos.	Pos.							Pos.	Pos.	Pos.
41	Neg.	Neg.	Pos.							Neg.	Neg.	Neg.
43	Pos.	Pos.	Pos.							Neg.	Neg.	Pos.

*Stuart's medium.

NE = Not examined.   Neg. = Negative for leptospires.   Pos. = Positive for leptospires.

TABLE IV. Results of Isolation of <u>Leptospira</u> <u>canicola</u> (Strain Moulton Dog Clone 36 HP) from Canine Urine in Stuart's Medium.

Number of leptospires inoculated	Number of tubes inoculated	Number of tubes positive[*]	Number of tubes negative[**]	Number of tubes contamin-ated
1.5×10^7	12	11	0	1
1.2×10^7	12	10	1	1
9×10^6	12	11	0	1
6×10^6	21	13	5	3
4×10^6	21	17	0	4
2×10^6	21	17	0	4
1.5×10^6	6	6	0	0
10^5	6	6	0	0
5×10^4	6	6	0	0
4×10^4	9	6	0	3
2×10^4	9	7	0	2
10^4	9	7	0	2
8×10^3	9	8	0	1
4×10^3	9	7	0	2
2×10^3	9	6	0	3
1.5×10^3	9	6	0	3
10^3	9	9	0	0
5×10^2	9	8	0	1
4×10^2	9	6	2	1
2×10^2	9	6	2	1
10^2	9	6	3	0
40	11	7	4	0
20	11	4	7	0
10	11	7	4	0

[*]Growth of leptospires in culture medium.

[**]No growth of leptospires in culture medium.

TABLE V. Results of Darkfield Microscopy and Fluorescent Antibody Techniques (FAT) on Canine Urine Containing Leptospira canicola (Strain Moulton Dog Clone 36HP).

Undiluted leptospires count	Dilution	Number of leptospires per ml.	Darkfield examination	FAT
1.8×10^8/ml	$1{:}18 \times 10^3$	10^4	Pos.*	Pos.
	$1{:}36 \times 10^3$	5×10^3	Pos.	Neg.**
	$1{:}72 \times 10^3$	2.5×10^3	Pos.	Pos.
	$1{:}18 \times 10^4$	10^3	Neg.	Neg.
	$1{:}36 \times 10^4$	5×10^2	Neg.	Neg.
	$1{:}72 \times 10^4$	2.5×10^2	Neg.	Neg.
	$1{:}18 \times 10^5$	10^2	Neg.	Neg.
	$1{:}18 \times 10^6$	10^1	Neg.	Neg.
	$1{:}36 \times 10^6$	5	Neg.	Neg.

*Pos. = Positive for presence of leptospires.

**Neg. = Negative for presence of leptospires.

TABLE VI. Results of Positive Control Urine Solids Smears Containing Leptospires and Spermatozoa Stained by Fluorescent Antibody Techniques with Conjugates Previously Adsorbed With Acetone Dried Tissue Powders.

Dilution of the adsorbed conjugate	Number of slides stained with each tissue powder adsorbed conjugate	Mouse liver powders	Dog liver powders	Dog testis powders
		Results	Results	Results
1:4	4	Spermatozoa stained bright yellowish-green.	Spermatozoa stained bright yellowish-green.	Heads of spermatozoa stained faint yellowish-green.
1:8	4	Similar findings.	Similar findings.	Similar findings.
1:16	4	Similar findings.	Similar findings.	Similar findings.
1:36	4	Similar findings.	Similar findings.	Similar findings.

EXPLANATION OF PLATE I

Fig. 1. Leptospires in canine (from clinic) urine (cellulose
filter membrane smear). Fluorescent antibody conju-
gate (adsorbed with canine testis tissue powders);
X 2000.

Fig. 2. Leptospira canicola (Strain Moulton Dog Clone 36HP).
Canine urine sediment (control). Fluorescent anti-
body conjugate (unadsorbed).
Note--the nonspecific fluorescence of the spermatozoa (a)
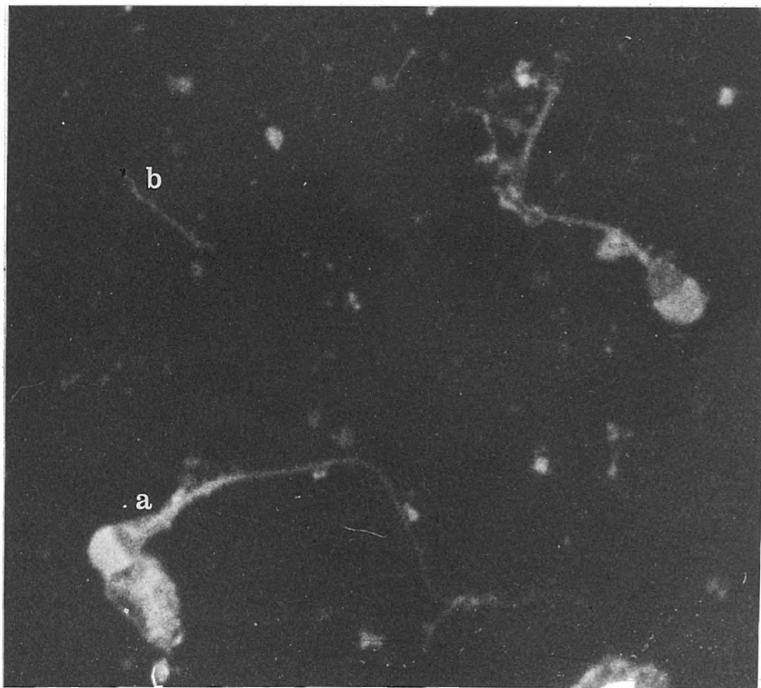and the specific fluorescence of the leptospirum (b);
X 1500.

PLATE I

Fig. 1

Fig. 2

EXPLANATION OF PLATE II

Fig. 1. Canine urine sediment. Fluorescent antibody conjugate
         (unadsorbed).
         Note--the nonspecific fluorescence of the spermatozoa tail
         fragments; X 2000.

Fig. 2. Canine urine sediment (control). Fluorescent antibody
         conjugate (adsorbed with mouse liver tissue powders).
         Note--the nonspecific fluorescence of the spermatozoan
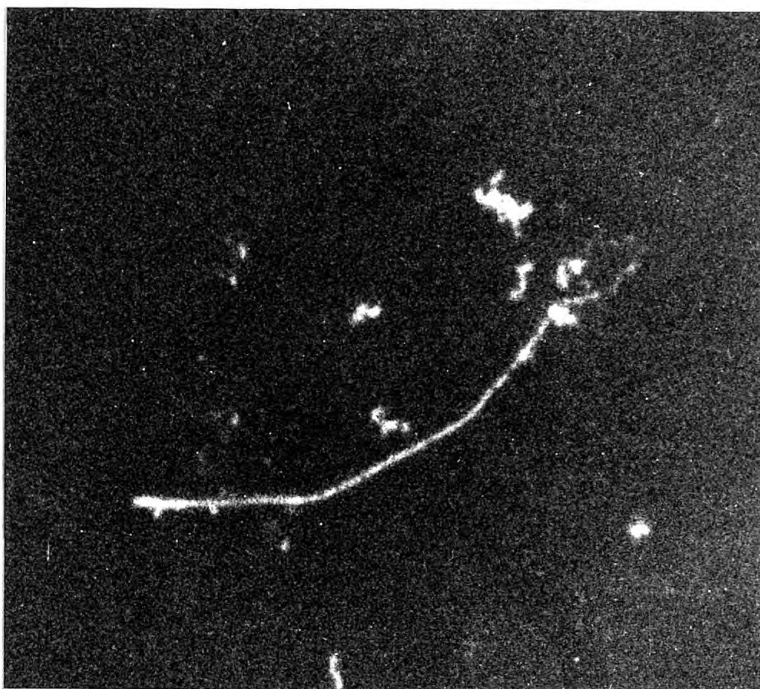   tail portion; X 2000.
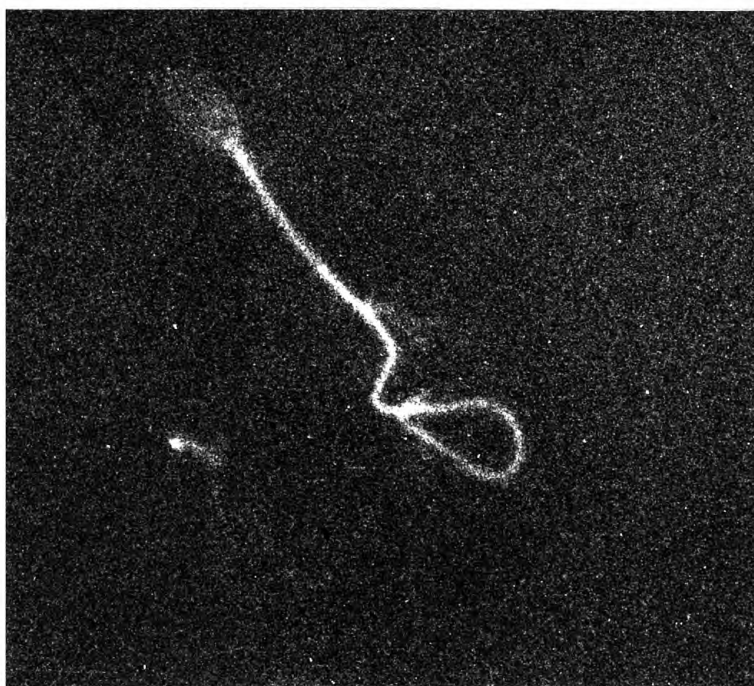
PLATE II

Fig. 1

Fig. 2

EXPLANATION OF PLATE III

Fig. 1. Canine urine sediment. Fluorescent antibody conjugate
         (adsorbed with dog liver tissue powders).
         Note--the faint nonspecific fluorescence of the
         spermatozoan tail portion; X 2500.

Fig. 2. Leptospira canicola (Strain Moulton Dog Clone 36HP) in
         control smear of canine urine sediment. Fluorescent
         antibody conjugate (adsorbed with dog testis tissue
         powders).
         Note--the minimum nonspecific fluorescence of the
         spermatozoan and the specific fluorescence of the lepto-
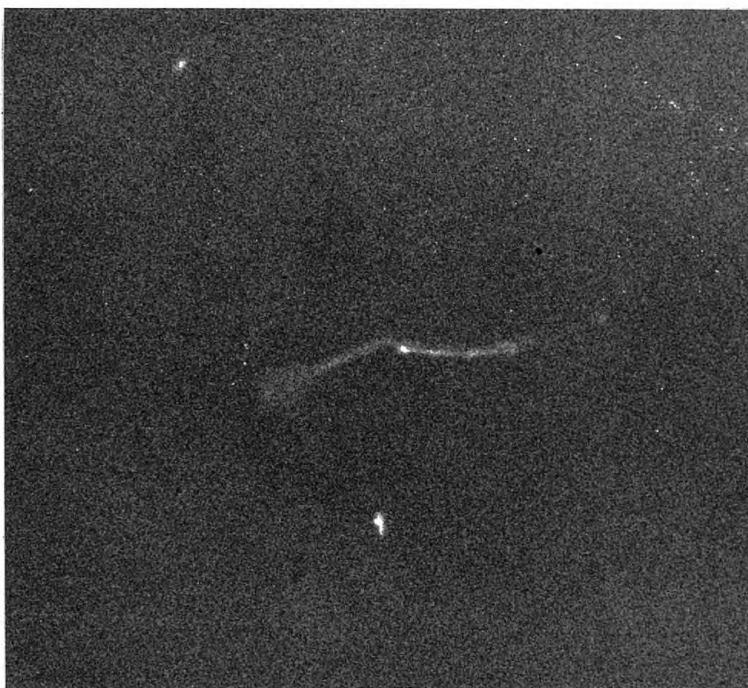   spirum lying beside the tail portion; X 2500.

PLATE III

Fig. 1

Fig. 2

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HUMORAL ECOLOGY OF LEPTOSPIROSIS IN DOGS

by

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Leptospirosis is the world's most widespread contemporary zoonosis. Many species of domestic and wild animals act as sources of infection to susceptible animals and man. Infection in man is closely related to the incidence in animals. Several reports of surveys done in dogs in the United States indicated an average incidence of 12%. Canine leptospirosis has not been surveyed in Kansas for some time; consequently this present study was initiated.

The objectives of this study were to (1) randomly survey urine specimens from dogs brought to the Dykstra Veterinary Clinic, Kansas State University, for possible leptospires shedders by fluorescent antibody techniques; (2) determine the minimum number of leptospires in dog's urine which can be detected by darkfield microscopy, fluorescent antibody techniques and recovered by culture; and (3) satisfactorily control nonspecific staining of urine solids including spermatozoa.

Urine specimens of 591 dogs, including street dogs, brought to the Dykstra Veterinary Clinic between November 1970 and August 1971 were surveyed for possible leptospires shedders by fluorescent antibody and culture techniques.

Midstream urine was collected from dogs while exercised. One drop of urine was immediately inoculated into 5 ml of Stuart's medium. The urine pH was adjusted to 7.2 to 7.6. In the laboratory 15 ml of urine were centrifuged at 381 g (1,500 RPM) in Sorvall G-L-C-1 centrifuge for 15 minutes. Two ml of supernatant were then filtered through a Millipore filter membrane and 4-6 drops of filtrate were inoculated into Stuart's medium. The remaining portion of the supernatant was formalinized. The tubes were incubated at $30^{\circ}C$ and

examined for growth of leptospires under darkfield. They were held for 4 weeks before they were considered negative. Smears were made from Millipore filter membranes on slides and stained with fluorescein labeled antibody (FAT) against Leptospira canicola (Strain Moulton Dog Clone 36HP) and examined. Specimens from 19 (3.1%) dogs proved positive for leptospires by FAT on filter membrane smears but were not detected in formalinized urine specimens. Attempts to recover leptospires from urine samples were unsuccessful.

In order to assure validity of the techniques employed, 4 dogs were experimentally infected with L. canicola (Strain Moulton Dog Clone 36HP). Leptospires were recovered from blood of all 4 dogs during the febrile period and all became shedders 9-16 days post inoculation. Leptospires were recovered from urine of 3 dogs in culture media. Leptospires were demonstrated in smears from Millipore filter membranes and formalinized urine specimens.

In studies relating to numbers of leptospires, 5-7-day-old cultures of L. canicola with known leptospires counts were serially diluted in Stuart's medium and the final dilution was made in Millipore filtered fresh urine. Known numbers of leptospires starting with 1.5×10^7 were inoculated into 5 ml Stuart's medium and incubated at 30°C. They were examined under darkfield at weekly intervals. Leptospires were successfully recovered by culture techniques in concentration of 10 organisms in 5 ml medium. Culture technique was compared with darkfield microscopy and FAT. A 5-7-day-old culture with known leptospires counts was serially diluted in fresh urine. One to three drops from each final dilution were examined under darkfield for leptospires. The darkfield examination was considered positive only when motile leptospires were seen. A 5 ml sample of each final dilution was then centrifuged at 1,522 g

(3,000 RPM) in Sorvall G-L-C-1 centrifuge for 45 minutes. The smears from the sediments were stained by FAT.

It was found that FAT and darkfield examination were equally effective on fresh specimens. Leptospires were detected by both methods in concentrations of leptospires as low as 2,500 organisms per ml.

Nonspecific fluorescence of spermatozoa, especially the disintegrated tail fragments, was evident during the survey. Acetone dried mouse liver, dog liver and dog testis tissue powders were evaluated to eliminate this nonspecific fluorescence. Nonspecific fluorescence could be satisfactorily eliminated only by dog testis tissue powder adsorption.