STUDIES ON CANINE LEPTOSPIROSIS: I. EVALUATION OF LABORATORY DIAGNOSTIC PROCEDURES. II. SEROLOGICAL DETERMINATION OF THE INCIDENCE OF LATENT INFECTION IN THE LANSING MICHIGAN AREA

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INTRODUCTION

Prior to July, 1945, canine leptospirosis had only been clinically diagnosed in the Lansing, Michigan, area. At this time a dog demonstrating icterus was admitted to the Veterinary Clinic at Michigan State College. The animal died 12 hours after presentation. An autopsy was performed, and the lesions were highly suggestive of leptospirosis. A guinea pig was inoculated intraperitoneally with the blood of the dog's heart and developed icterus and died 9 days after inoculation. Leptospirae were demonstrated in the kidney tissues by darkfield examination and sections stained by a silver impregnation method. Cultures were also obtained and serologically the organism appeared to be Leptospira canicola.

Because of the similarity in clinical symptoms of canine leptospirosis, dog distemper, black tongue, and various gastro-intestinal disturbances, the clinician is often unable to make a differential diagnosis. As a result, the immediate instigation of specific therapeutic measures is not possible.

The literature revealed that many laboratory procedures are recommended as aids in the diagnosis of leptospirosis. However, an evaluation of them was not available. Experiment I was planned to evaluate the relative merits of the several recommended laboratory procedures for a rapid, accurate diagnosis of leptospirosis.

Canine leptospirosis appeared to be more prevalent in the ensuing two years than previously. The epizootological possibility that many dogs in this area were excreting the organisms in their urine, thus serving as sources of infection to dogs and man, was considered of importance.

Meyer and coworkers (1939a) reported that sera obtained from ten dogs in the Detroit, Michigan, area were serologically positive to L. icterohaemorrhagiae, the causative agent of Weil's disease in man. Since that time no further studies in Michigan have been reported.

To add to Meyer's data relative to the incidence of the disease in Michigan and to determine the incidence of latent canine leptospirosis in the Lansing, Michigan, area, Experiment II was planned.

HISTORICAL REVIEW

Disease

Twenty-eight years after Weil described the disease in man, which bears his name, Inada and coworkers (1916), who experimentally transmitted the disease to guinea pigs in 1913, isolated and cultured the causative agent, which they named Spirochaeta icterohaemorrhagiae. Unacquainted with the work of Inada, Huebener and Reiter (cited by Walch-Sorgdrager, 1939) and Uhlenhuth and Fromme (1915) transmitted the disease to guinea pigs and named the organism Spirochaeta nodosa and Spirochaeta icterogenes, respectively. Noguchi (1917) isolated the same organism from wild rats in New York City and, because of its fine minute windings and dissimilarity to other spirochetes, proposed the name, Leptospira icterohaemorrhagiae.

That Weil's disease (leptospirosis) may occur in the canine has been suspected for some time. Krumbein and Frieling (1916) reported an unconfirmed case of an association of two humans with a case of canine jaundice, later developing typical Weil's disease. Courmont and Durand (1917) demonstrated puppy (canine) susceptibility to Leptospira icterohaemorrhagiae by subcutaneous and intraperitoneal inoculation or ingestion of infectious liver material, although Nicolle and Lebailly (1918) were unsuccessful in their attempts.

Perhaps the first to suspect that there was another species of Leptospira which may infect dogs were Lukes (1924) and Krivacek (1924), who demonstrated a spirochete in the tissues of dogs, which had died of canine typhus or Stuttgart's disease, and called it Spirochete melanogenes canis.

Okell, Dalling and Pugh (1925) proved that infectious jaundice, occurring naturally in dogs, was caused by a spirochete. They were able to isolate 3 strains of the organism, which corresponded in every way to L. icterohaemorrhagiae obtained from rats. It was suggested that rats transmitted the disease to dogs by contaminating the food and bedding with their body excretions and that the organism may be found in the urine of the dog as well as of man.

The first to recognize and describe the symptoms and pathology of canine leptospirosis, as caused by a new species, were Klarenbeek and Schuffner in 1931 (1933). The species is now known as L. canicola. The strain isolated was agglutinated by specific serum to a titer of 1:10,000 and with serum specific for L. icterohaemorrhagiae to a titer of 1:25. The epidemiological importance of this discovery was emphasized by Klarenbeek (1934), who demonstrated that L. canicola may also cause infection in man.

/Reports of the occurrence of canine leptospirosis in the United States have been comparatively recent. Jungherr (1937) appears to be the first to report the canine disease in the United States. He reported an outbreak in a pointer kennel in which 41 of 42 dogs, less than 1 year of age died of the disease., Employing Levaditi's silver stain, spirochetes were demonstrated in the liver, lung and kidney tissue, and the Lieberkuhun glands of the colon. Although guinea pig inoculations and subcultures were initially successful, the strain was eventually lost. The epizootological data indicated that the outbreak was caused by L. icterohaemorrhagiae. However, the organism's virulence for the guinea pig was characteristic of L. canicola.

Meyer and coworkers (1938) were the first to isolate L. canicola from dogs in this country. Two cases of human canicola infection were also reported by these authors. Their darkfield examination of wild rat kidneys indicated that 33 per cent harbored the organism. Meyer and coworkers (1939a) published an excellent review of their studies on all aspects of the canine disease in 1939.

The incidence of latent canine leptospirosis in Pennsylvania was reported by Raven (1941), whose results indicate that 28-38 per cent of the animals examined had apparently been infected at one time. Greene (1941) employed the macroscopic agglutination test and reported the results of his survey conducted in southern California. Twenty-nine per cent of the dogs he examined were serologically positive to L. canicola and none to L. icterohaemorrhagiae. One of a hundred feline and 2 of 426 human specimens demonstrated a positive reaction to L. icterohaemorrhagiae; all were negative to L. canicola.

Elander and Perry (1940) and Bloom (1941) presented a study on 6 and 9 cases of canine leptospirosis, respectively. Bloom emphasized that only 5 of the 9 cases he observed demonstrated jaundice, and discussed in detail his pathological findings.

The first isolation of L. icterohaemorrhagiae from a canine in the United States was reported by Randall (1944). He employed the golden hamster, following the recommendations made by Morton (1942) that the golden hamster (Cricetus auratus) was the animal of choice for the isolation of Leptospira.

Serological tests

The adhesion, complement-fixation, and agglutination-lysis tests have been employed in the serological identification of Leptospira and

diagnosis of infection. The application of these tests has greatly enhanced the studies conducted in the past and provided a firm foundation for future observations.

Laveran and Mesnil (1901) demonstrated the fact that particles; such as, blood platelets or bacteria, become adherent to trypanosomes or spirochetes in the presence of their own immune serum. Brown and Davis (1927) described the uses of the thrombocytobrin or Rieckenberg reaction and proposed the name, "adhesion test." The authors concluded from their experiments that L. icterohaemorrhagiae and L. icteroids were identical and in addition they serologically examined 100 London wild rats. Brown (1935) demonstrated that the test could be employed in the serological diagnosis of Weil's disease in man.

Noguchi (1920) was the first to carry out the complement-fixation test with Leptospira antigen and obtained positive results. He employed the test in his comparative investigations between L. icteroides and L. icterohaemorrhagiae strains. It was proven that a specific complement fixation in Weil's disease was possible, although fixations were only achieved with 0.1 ml immune serum.

Bessemans and Nelis (1928) tried the complement fixation test with Leptospira antigen, which had been concentrated by centrifugation and suspended in physiological salt solution. Employing this as antigen, they obtained positive fixation with 3 sera from human patients and 1 serum from a dog with Weil's disease. They also demonstrated that positive Wasserman-reacting sera gave negative complement-fixation reactions with Leptospira antigens.

Pot and Dornick (1936) examined 100 human sera, employing the complement fixation and agglutination-lysis test. Although the aggluti-

nation lysis test consistently gave higher titers, the complement-fixation test was recommended as a reliable diagnostic procedure for Weil's disease.

The effect of immune serum (convalescent serum from patients with Weil's disease) on cultured spirochetes was initially discussed by Ito and Matsuzki (1916). They state that the sera had a "slight bactericidal action." Martin, Pettit, and Vaudremer (1917), who first employed Leptospira cultures in serological diagnosis, reported that serum from a patient with Weil's disease agglutinated Spirochetae icterohemorrhagiae in 1:500 dilution. Normal and syphilitic sera were negative.

Schuffner and Mochtar (1927) gave a very thorough account of the agglutination-lysis reaction and suggested the important point that lysis of leptospirae takes place without the influence of complement. They observed that agglutination takes place only in the low serum dilutions with lysis occurring in the higher dilutions. It was also observed that there will always be some leptospirae which escape the lytic process in those dilutions where this takes place.

The use of formalin-killed cultures in the agglutination test was reported by Walch-Sorgdrager (1939). He observed that only agglutination occurred when the killed culture was employed. The titer obtained by this method was equal to that obtained employing the living organism in the lysis test.

The first macroscopic agglutination test was described and employed by Pot (1936), who examined sera from 26 patients positive to Schuffner's agglutination test. Twenty-five of the 26 gave corresponding results; one was negative.

Smith and Tulloch (1937) reported on a macroscopic plate agglutination test and grew the organism in Schuffner's media, using it in the live state. Having previously demonstrated that 55 C for 5-30 minutes and .1-5 per cent formalin altered the morphology of the organism, the authors believed that this would be alleviated if the organism was first sensitized with its specific antibody. This proved to be true and, upon incubation of the serum-antigen dilutions at 37 and 55 C, floccules were easily observed with a hand lens and no difficulty was experienced in interpreting the results. All human and guinea pig sera examined gave almost identical reactions with the macroscopic plate agglutination test as those observed with Schuffner's agglutination-lysis test.

Brown (1939) employed the rocking slide macroscopic agglutination test and recommended it for use as a rapid presumptive serological test for Weil's disease. The rocking slide method and the macroscopic method of Pot gave consistently identical results. Schuffner's technique, which was employed in conjunction with these two, gave titers three times higher.

Starbuck and Ward (1942) made a comparison of the macroscopic plate agglutination test and the standard microscopic agglutination test, employing 356 human sera. The L. icterohaemorrhagiae antigen for the macroscopic test was supplied by Joyner and consisted of a concentrated suspension of leptospirae, to which gentian violet had been added. They reported that the microscopic test was more accurate but believed the macroscopic plate test sufficiently specific and sensitive to be of value in the diagnosis of leptospiral jaundice in humans.

METHODS AND MATERIALS

Preparation of materials for microscopic, cultural, and serological studies

Blood and urine, obtained from normal and Leptospira-infected dogs, were employed as the original materials on which microscopic, cultural, and serological examinations were conducted.

The equipment employed in the collection of the above materials was sterilized by autoclaving at 15 lbs. pressure for 45 minutes or by dry heat at 180 C for 4 hours.

Microscopic examinations conducted on stained sediment and dark-field preparations were made, employing the 10X ocular and 97X objective.

Observing aseptic precautions, 8-10 ml of blood was drawn from the radial or jugular vein of each dog with a sterile syringe and needle. The blood was then expelled into a sterile rubber-stoppered, 30 ml vaccine vial containing 8-12 glass beads and completely defibrinated by shaking.

The vials, containing the defibrinated blood, were then centrifugalized at 1,600 rpm for 10 minutes to remove the blood cells. The serum was then removed aseptically with a sterile 5 ml pipette and expelled into a second sterile vial and recentrifugalized at 2,800 rpm for 1 hour. The second centrifugation, the purpose of which is to throw down the leptospirae, was conducted only on the samples intended for microscopic and cultural studies. The serum, employed in the serological studies, was placed in a third sterile vial, and 0.01 ml of the sediment was placed on each of 3 glass slides. One slide was

stained with Giemsa's stain, one with Fontana's, and the third used for darkfield examination.

The urine specimens were collected by catheterization as aseptically as possible. The external genitaliae were cleansed with Roccal and care exercised in introducing the catheter to avoid contamination prior to insertion into the urethral opening. Wherever possible 20-30 ml were collected in sterile 30 ml vaccine vials.

Two centrifugation procedures were conducted on the urine specimens and 3 slides prepared for examination as was done with the blood sediments.

The Giemsa staining procedure, as outlined by Stavitsky (1945), was conducted on one blood and one urine sediment slide from each dog. The stain was prepared by adding, to 50 ml of boiled distilled water, 2 drops of 0.5 per cent sodium bicarbonate, 1.5 ml methyl alcohol, and 1.25 ml of Giemsa stain[1] (stock solution). All smears were allowed to dry overnight, fixed in methyl alcohol for 3 minutes, and then stained with the prepared Giemsa stain for 12-16 hours. Positive slides were employed as controls in conjunction with each group of slides stained. In the Giemsa preparations the organisms stained reddish-violet and the background, a lighter pinkish-blue.

The urine and blood sediment slides, that were stained by Fontana's method, were also allowed to dry on the slide overnight. Fixation was accomplished by placing the slides in Ruge's acetic acid-formalin-water solution for 2 minutes. The staining procedure, employing Fontana's tannic acid mordant and silver nitrate solutions, was then completed and the preparations examined by microscopy. Positive slides were em-

[1]National Analine and Chemical Co., Inc., New York, New York

ployed as control. By this method the organisms stain black against a light-brown background.

Prepared slides of the centrifugalized blood and urine sediments were also examined by darkfield illumination. One-tenth milliliter of each sediment was placed on a clean glass slide and immediately covered with a No. 1, 22-mm square cover glass. Care was exercised to prevent the occurrence of air bubbles in the preparation, for they reflected the light, making the examination for Leptospira difficult. The edges were then sealed with paraffin to prevent evaporation, and a careful microscopic examination was made for 15 minutes, employing the 10X ocular and 97X objective. A second preparation for darkfield examination was made of all specimens in which suspicious leptospirae were observed. The organisms in these preparations appeared as bright silver threads against a black background.

Isolation of the organism

Schuffner's modification of Verwoort's medium (cited by Meyer, 1939) was used exclusively in the cultural studies conducted on blood and urine sediments and the production of antigen. Nine milliliter amounts were dispensed in 20X175 mm culture tubes to which 1 ml of sterile, inactivated, rabbit serum was added prior to use. Rabbit serum known not to contain antileptospiral substances was used.

The remaining blood and urine sediments were suspended in 2 ml of Schuffner's medium (minus rabbit serum). One milliliter amounts of this suspension were used to inoculate 2 tubes of Schuffner's medium. The proportions of sediment to media were 1:10-1:20 and yielded the best results by trial experiments.

All cultures were incubated at 30 C with periodic macroscopic and darkfield examinations performed to determine the presence or absence of growth. Three transfers were made at 7-day intervals to new culture medium, regardless of the results of these examinations. All cultures were held for 4 weeks before being discarded as negative.

Preparation of antigens

The antigens were made from cultures of L. canicola and L. icterohaemorrhagiae, which were obtained through the courtesy of Dr. K. F. Meyer of the George Williams Hooper Foundation.

To make it possible to employ freshly-prepared antigen, antigen production was continued throughout the experiment.

It is necessary that a medium supporting heavy growth of leptospirae, in an otherwise clear medium, free from clumps and particulate matter, be employed for antigen production. Trials were made with the various media as recommended by Noguchi and Battistini (1926), Korthof (1932), Starbuck and Ward (1942), and Chang (1947); however Schuffner's modification of Verwoort's medium was found to be far superior to the rest.

The following slight modifications were made in Schuffner's medium, adapting it to our use:

1. It was found that filtration of the supernatant fluid through a No. 6 Seitz filter, employing a type ST disc[1], gave the same results as a Berkefeld filter of carifying porosity.
2. The growth of the Leptospira is dependent to a great de-

[1] Hercules Filter Corporation, Paterson, New Jersey

gree upon the pH of the medium. The original recommendations that the pH fall in the 6.8-7.2 range allows too wide a pH variation. A pH of 7.15-7.25 was found to be more conducive to growth of both species of Leptospira.

3. The medium was tubed and autoclaved immediately.
4. Inactivated rabbit serum was added to the medium prior to use.

For antigen production 0.5 ml of each stock culture was pipetted into a number of culture tubes containing 10 ml of Schuffner's medium. Inoculated tubes were incubated at 30 C for 4-5 days. At this time the organisms were still young and vigorously growing. Macroscopic and microscopic examinations were made of all antigen tubes to check growth, morphology, and the presence of bacterial contamination. All tubes, in which atypical morphology, poor growth, or in which bacterial contamination was observed, were discarded.

The antigens employed in the agglutination test were prepared by adding 0.15 per cent neutral formalin to each tube. The tubes were then centrifugalized for 10 minutes at 1,600 rpm to remove any clumps of organisms which might interfere with the interpretation of the agglutination reaction.

The sensitivity of the antigen was checked by employing known negative and positive sera of varying titers. Those antigen suspensions yielding satisfactory results were then stored in the refrigerator.

Positive and negative sera

Positive and negative inactivated rabbit sera were used as controls in the agglutination test. Negative sera were also added to Schuffner's medium, 10 per cent by volume, to enhance growth.

The negative control sera were obtained from normal rabbits and examined by the agglutination test for the presence of agglutinins. The negative sera to be added to Schuffner's medium were examined by cultural methods in addition to the agglutination test.

The cultural examination consisted of adding 1 ml of each serum to be tested to each of 4 tubes of culture media. Two tubes were inoculated with L. canicola and 2 with L. icterohaemorrhagiae, as outlined in the procedure for antigen production. Microscopic darkfield counts were made on the first, third, and fifth days of incubation and all tubes examined macroscopically and microscopically at the end of the incubation period. Any negative serum demonstrating inhibition of growth or variability in morphology was discarded.

Positive control sera were produced according to the following procedure. Vaccines were prepared from 6-day-old cultures of each species, to which 0.15 per cent neutral formalin was added to fill the organisms. Three 2-ml. injections of the killed preparations were given intravenously into the marginal ear vein of a rabbit every 5th day. A final injection, consisting of 2 ml of the live culture, was administered on the 15th day.

Antibody production in the rabbits took place with almost automatic regularity. On the 5-6th day after the first injection the titer was approximately 1:400; on the 12th day the titer was 1:3,000; on the 18th day the titer was 1:6,000-12,000; on the 24th-30th day the titer was 1:24,000-30,000. The titer will fall gradually (if additional injections are not given), and in the case of the animals examined, the titer after 5 months was down to 1:300-400. Although individual rabbits will vary somewhat to antigenic response, 7 of the 8 rabbits employed for positive

sera production gave the typical reaction as outlined above. "Booster shots" were administered periodically, usually consisting of a 2-ml injection of the formalin-killed culture and a 2-ml injection of the living culture at weekly intervals.

It might be of interest to point out that the first sign of antibody in the serum does not appear until the 4th day after the first 2 ml of vaccine is administered. On the 4th day the agglutination reaction is negative, on the 5th day agglutination can be detected in dilutions of 1:100-300.

All positive and negative control sera were filtered through germicidal discs[1], using a No. 3 Seitz filter, inactivated at 56 C for 30 minutes prior to storage in the refrigerator. It was noted that the titer of the positive sera dropped during storage; therefore fresh bleedings were made every month.

Agglutination test

The agglutination test, as outlined by Schuffner and Mochtar (1927), was conducted on all sera examined. The macroscopic plate test, as recommended by Brown (1939), was used in trial experiments and found not to be as sensitive as the microscopic test originally devised by Schuffner.

Serum dilutions of 1:5, 1:50, 1:500, and 1:5,000 were made in 12X 75 mm tubes according to the following table:

Tube	1	2	3	4
Buffer*	1.2 ml	0.9 ml	0.9 ml	0.9 ml
Serum	0.3 ml**	0.1 ml**	0.1 ml**	0.1 ml
Dilution	1:5	1:50	1:500	1:5,000

*The buffer consisted of Schuffner's medium minus rabbit serum.
**0.1 ml is transferred from tube 1 to 2, 2 to 3, and 3 to 4.

[1]Central Scientific Company, Chicago, Illinois

Serum-antigen dilutions were also made in 12X75 mm tubes in duplicate sets, employing L. canicola as the antigen in one set and L. icterohaemorrhagiae in the other according to the following table:

Tube	Serum	Antigen	Buffer	Dilution
1	0.15 ml of 1:5	0.15 ml	0	1:10
2	0.15 ml of 1:5	0.15 ml	0.10 ml	1:30
3	0.15 ml of 1:50	0.15 ml	0	1:100
4	0.15 ml of 1:50	0.15 ml	0.10 ml	1:300
5	0.15 ml of 1:500	0.15 ml	0	1:1,000
6	0.15 ml of 1:500	0.15 ml	0.10 ml	1:3,000
7	0.15 ml of 1:5,000	0.15 ml	0	1:10,000
8	0.15 ml of 1:5,000	0.15 ml	0.10 ml	1:30,000
9	None	0.15 ml	0.15 ml	Control*

*One set of each positive and negative control sera was set up with each series of tests conducted.

After the serum-antigen dilution tubes were shaken for 5 minutes and incubated in a water bath for 3 hours at 37 C, 1 loopful of each dilution was placed on a clean glass slide and covered with a No. 1, 22-mm square, cover glass. All slides were examined by darkfield illumination, employing the 10X ocular and the 10X, 43X, and if the reactions appeared doubtful, the 97X objective.

Interpretation of the agglutination reactions

The varying degrees of agglutination observed were arbritarily given plus values to standardize the readings as follows: 1 plus, small clumps of 3-10 leptospirae and many free ones; 2 plus, numerous small clumps of leptospirae and many free ones; 3 plus, numerous large clumps of leptospirae and few free ones; 4 plus, numerous large clumps of leptospirae and only an occassional free one. Sera, demonstrating a partial reaction in the lower serum-antigen dilutions, were retested.

Difficulty was encountered in properly evaluating the agglutination reactions which occurred in the lower serum-antigen dilutions. As yet, the frequency and possible limits of nonspecific reactions have not been sufficiently investigated. It is possible that the low titer reactions may indicate a recent or previous leptospiral infection or may be entirely nonspecific in nature.

RESULTS OF EXPERIMENT 1

In an attempt to evaluate some of the laboratory diagnostic procedures, micorscopic, cultural, and serological examinations were conducted on the blood and microscopic and cultural examination on the urine of 30 naturally-infected dogs. The results of this evaluation are summarized in Table 1.

Microscopic blood and urine sediments, stained by Giemsa's and Fontana's methods, were conducted. Table 1 shows that the number of speciments, in which leptospirae were detected by these methods, were very small. None of the Giemsa blood preparations and only 1 (3.33%) of the urine preparations demonstrated Leptospira. Three (10%) of the Fontana blood preparations and 1 (3.33%) of the urine preparations demonstrated Leptospira.

In addition to the above two microscopic procedures, darkfield examinations were made. In 7 (23.33%) of the blood preparations and 1 (3.33%) of the urine preparations, Leptospira appeared to be present.

The urine sediment from the same dog was positive in all 3 of the microscopic examinations.

The results of the cultural examinations of blood compare favorably with those obtained by darkfield examination. Isolations were obtained from 6 (20%) of the samples cultured. All the organisms cultured appear to be L. canicola as evidenced by serological examination.

The results, obtained by attempting to culture Leptospira from urine sediment, were all negative. Unfortunately, it was impossible to culture urine sediment without bacterial contamination; as a result this diagnostic procedure was considered unreliable.

The results of the 3 serological tests conducted at 6-8 day intervals on the 30 infected dogs are summarized in Table 2. The results recorded for the first test designate the highest dilution in which agglutination was observed. For the second and third tests, only the dilutions, in which a 4-plus agglutination reaction in the 1:100 serum dilution, or higher, was considered of diagnostic significance.

Sixteen (53.33%) of the animals tested were males. The ages for all the animals tested ranged from 6 months to 7 years.

On the initial test, 14 (46.66%) demonstrated an agglutination reaction to one or both antigens. However, all of the 30 samples demonstrated some degree of agglutination on the second and third tests.

Of the 14 sera which demonstrated some degree of agglutination in the initial test, only one yielded a 4-plus reaction in the 1:100 serum dilution. The results of the second test indicate that 16 of the sera yielded 4-plus reactions in the 1:100 serum dilution, whereas on the third test all gave 4-plus reactions in the 1:100 serum dilution or higher.

The degree of agglutination observed in the first, second and third tests varies considerably. They range from negative to 4 plus in 1:100, 4 plus in 1:10-10,000, and 4 plus in 1:100-30,000, respectively. As was expected, the titers rose as the disease progressed, the strongest reactions in the higher dilutions occurring in the third test.

It should not be assumed that an end titer of 1:30,000 (Table 2) is the highest titer that may be expected. Other examinations, not recorded here, reveal that the titer may reach at least 1:300,000.

Arbritarily, the infected dogs were divided into 3 age groups, as follows: 1 month to 1 year, 1 year through 3 years, and 4 years through

7 years. Nine (30%) of the dogs were in the 1st group, 14 (46.66%) in the second, and 7 (23.34%) in the third group, respectively. None of the animals in this study were over 7 years of age; in fact, the author is yet to observe a clinically active case of the infection in dogs over 7 years old.

The occurrence of specific and paraspecific reactions are summarized in Table 3. These results are based on the reactions observed for each antigen in the third agglutination test.

It was noted that only 10 (33.33%) of the sera failed to demonstrate any paraspecific reactions with L. icterohaemorrhagiae antigen. Furthermore, as the specific titer increased, there was also an increase in degree and frequency of the paraspecific reactions.

It has been reported by Meyer and coworkers (1939), Raven (1941), Walch-Sorgdrager (1939), that infections due to L. canicola yield higher and stronger reactions than those due to L. icterohaemorrhagiae. The results of the third test indicate that 22 (73.33%) of the sera demonstrated 4-plus reactions in dilutions of 1:1,000 or higher.

TABLE 1

Comparative microscopic and cultural findings

in 9 positive dogs

Dog No.	Giemsa		Fontana		Darkfield		Culture	
	Bld.	U.	Bld.	U.	Bld.	U.	Bld.	U.
LP 1	-	-	-	-	+	-	+	-*
LP 3	-	+	-	+	-	+	-	-
LP 5	-	-	+	-	+	-	+	-
LP 8	-	-	-	-	+	-	-	-
LP 10	-	-	-	-	+	-	-	-
LP 15	-	-	+	-	+	-	-	-
LP 19	-	-	-	-	-	-	+	-
LP 21	-	-	+	-	+	-	+	-
LP 25	-	-	-	-	+	-	+	-

*All urine cultures became overgrown with bacteria.

TABLE 2

Serological results of 3 tests made at 6-8 day intervals

on 30 *Leptospira*-infected dogs

No.	1st Test	2nd Test	3rd Test	Sex	Age
LP 1	0* 0	0 4+, 1:10	0 4+, 1:300	M	8 mos.
LP 2	2+, 1:30 4+, 1:100	4+, 1:10 4+, 1:300	4+, 1:3,000 4+, 1:10,000	F	3 yrs.
LP 3	3+, 1:10 2+, 1:300	4+, 1:300 4+, 1:10,000	4+, 1:300 4+, 1:10,000	M	4 yrs.
LP 4	0 0	0 4+, 1:30	0 4+, 1:300	M	1 yr.
LP 5	0 0	0 4+, 1:30	0 4+, 1:100	F	6 mos.
LP 6	0 0	0 4+, 1:10	0 4+, 1:1,000	F	1 yr.
LP 7	1+, 1:10 0	4+, 1:10 4+, 1:10	4+, 1:100 4+, 1:300	F	6 mos.
LP 8	1+, 1:10 2+, 1:10	4+, 1:100 4+, 1:100	4+, 1:1,000 4+, 1:10,000	M	2 yrs.
LP 9	1+, 1:10 1+, 1:30	4+, 1:300 4+, 1:300	4+, 1:1,000 4+, 1:10,000	F	2 yrs.
LP 10	0 0	0 4+, 1:30	0 4+, 1:100	M	11 mos.
LP 11	3+, 1:30 3+, 1:100	4+, 1:300 4+, 1:1,000	4+, 1:3,000 4+, 1:30,000	M	7 yrs.
LP 12	4+, 1:30 3+, 1:30	4+, 1:100 4+, 1:100	4+, 1:100 4+, 1:3,000	M	3 yrs.
LP 13	3+, 1:10 3+, 1:10	4+, 1:10 4+, 1:30	4+, 1:3,000 4+, 1:10,000	M	1 yr.
LP 14	2+, 1:10 2+, 1:10	4+, 1:300 4+, 1:1,000	4+, 1:1,000 4+, 1:10,000	F	2 yrs.
LP 15	0 0	0 4+, 1:100	0 4+, 1:3,000	M	5 yrs.

*First line: titer of sero-reaction for *L. icterohaemorrhagiae* antigen; second line for *L. canicola*.

TABLE 2 (continued)

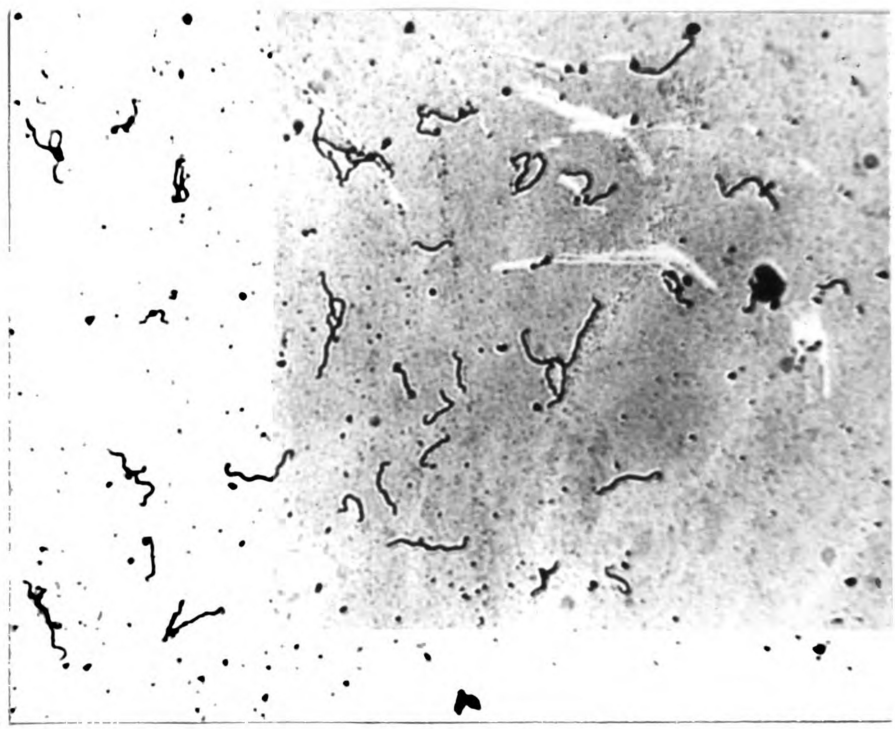
No.	1st Test	2nd Test	3rd Test	Sex	Age
LP 16	0 0	0 4+, 1:10	0 4+, 1:300	M	8 mos.
LP 17	1+, 1:10 1+, 1:10	4+, 1:10 4+, 1:100	4+, 1:30 4+, 1:1,000	F	4 yrs.
LP 18	0 1+, 1:30	0 4+, 1:30	0 4+, 1:1,000	M	1 yr.
LP 19	0 0	3+, 1:30 4+, 1:30	4+, 1:1,000 4+, 1:3,000	F	2 yrs.
LP 20	1+, 1:300 1+, 1:300	4+, 1:300 4+, 1:100	4+, 1:10,000 4+, 1:30,000	F	4 yrs.
LP 21	0 0	0 4+, 1:10	0 4+, 1:100	F	11 mos.
LP 22	0 0	4+, 1:300 4+, 1:300	4+, 1:3,000 4+, 1:10,000	M	2 yrs.
LP 23	0 0	4+, 1:100 4+, 1:100	4+, 1:300 4+, 1:3,000	F	6 yrs.
LP 24	0 0	4+, 1:30 4+, 1:100	4+, 1:100 4+, 1:1,000	F	10 mos.
LP 25	0 0	0 4+, 1:100	0 4+, 1:100	F	7 mos.
LP 26	3+, 1:10 2+, 1:100	4+, 1:100 4+, 1:100	4+, 1:1,000 4+, 1:3,000	M	1 yr.
LP 27	1+, 1:10 0	4+, 1:30 4+, 1:30	4+, 1:300 4+, 1:1,000	F	5 yrs.
LP 28	0 0	2+, 1:10 4+, 1:30	4+, 1:30 4+, 1:1,000	M	9 mos.
LP 29	0 0	2+, 1:30 4+, 1:30	4+, 1:100 4+, 1:1,000	M	3 yrs.
LP 30	0 0	4+, 1:100 4+, 1:3,000	4+, 1:300 4+, 1:3,000	M	1 yr.

TABLE 3

Titer of specific and paraspecific reactions in 30 dogs

(Active infection with L. canicola)

Specific L. canicola titer	Total No. Reacting	Paraspecific L. icterohaemorrhagiae titer							
		Neg.	10	30	100	300	1,000	3,000	10,000
100	4	4	0	0	0	0	0	0	0
300	4	3	0	0	1	0	0	0	0
1,000	6	2	0	1	2	1	0	0	0
3,000	6	1	0	0	1	2	2	0	0
10,000	8	0	0	1	0	1	3	3	0
30,000	2	0	0	0	0	0	0	1	1
Total	30	10	0	2	4	4	5	4	1



Leptospira canicola, 7-day culture from Schuffner's medium, Fontana stain, X 450. Isolated from canine, July, 1945.

RESULTS OF EXPERIMENT 2

In an attempt to ascertain the magnitude of reservoir leptospiral infection in the Lansing, Michigan, area, 5 ml of blood was drawn aseptically from the radial vein of 500 dogs. These animals were hospitalized in the Veterinary Clinic at Michigan State College or present in nearby kennels. At the time of bleeding, all dogs appeared normal, none showing any clinical evidence of leptospirosis. History relative to leptospirosis was unobtainable in most instances. Retests were not conducted on any of the dogs. For all specimens that were found unsatisfactory for any reason, second samples of blood were obtained.

All sera were inactivated in a water bath at 56 C for 30 minutes. This sera was stored in the refrigerator at 4 C until tested. The agglutination test was conducted on all samples within 3 days after collection.

Formalin-killed L. canicola and L. icterohaemorrhagiae antigens were employed in the agglutination test. A 1-plus reaction, or higher, in the 1:10 serum dilution or greater, was considered indicative of a latent infection.

A majority of the positive sera demonstrated a stronger reaction to one antigen than to the other. In these instances the antigen which gave the stronger reaction in the highest dilution was considered specific. The antigen giving the weaker reaction with the same serum was considered paraspecific.

Of the 500 canine sera examined, 148 (29.6%) gave a positive reaction to one or both antigens. The remaining 352 (80.4%) gave no reaction to either antigen. One hundred and thirty-one (26.2%) demon-

strated a stronger reaction with L. canicola antigen and 13 (2.6%) a stronger reaction with L. icterohaemorrhagiae antigen. Four of the samples (.8%) reacted with an equal intensity to both the L. canicola and L. icterohaemorrhagiae antigens. The details are given in Table 4.

Table 5 summarizes the incidence of leptospirosis among the various age groups. Arbritarily, age divisions were made dividing the group of dogs tested into four age groups. The groups were: 1 month to 1 year; 1 year through 3 years; 4 years through 7 years; and 8 years through 12 years. The incidence of infection in the various age groups was 26.90%, 17.40%, 42,60%, and 57.40% respectively. As is substantiated in Table 5, the greater the age the animal achieves, the greater the opportunity for exposure.

Attention is called to the 1 year through 3 year group (Table 5), which does not conform to this expected pattern. Two hundred and twenty-one of the 500 dogs, comprising nearly one-half of the animals tested, were in this group. Seventeen and forty-hundreths per cent of this group demonstrated Leptospira immune bodies. Attention is also called to the fact that 25.70% of the positive agglutination reactions occurred in this group. The reason for this could possibly be explained by the fact that kennel dogs were included in this experiment. The majority of their ages ranged from 1 year through 3 years. These young dogs were kept in kennels and runs with concrete floors. Rat infestation was not evident. They were not allowed to run free and roam as the pet dog. These kennel dogs had less chance of exposure to the infection than did the majority of other dogs tested.

It is possible that, if kennel dogs had not been included in this study, the number of positive animals would have been greater in this age group.

Two hundred and seventy-nine (55.80%) of the dogs tested were males, of which 104 (70.40%) gave a positive serological test to one or both antigens. Ninety-one (89.50%) of the male reactors were positive to L. canicola antigen, 10 (9.62%) to L. icterohaemorrhagiae, and 3 (2.88%) demonstrated an equal titer for both. Two hundred and twenty-one (44.20%) of the dogs tested were females, of which 44 (29.60%) gave a positive agglutination reaction to one or both antigens. Forty (90%) gave a positive reaction with L. canicola antigen, 3 (6.82%) to L. icterohaemorrhagiae, and 1 (2.28%) reacted with an equal titer to both. The percentage of male to female reactors was slightly more than 2:1.

The results (see Table 6), relative to the sex incidence of the disease, is in accord with the findings of Meyer (1939) and Raven (1941). Their examinations also dealt predominantly with L. canicola infections.

Klarenbeek (1934) reports that leptospirosis may occur up to 10 times as often in the male as in the female. The results indicate that L. icterohaemorrhagiae infections are predominate.

Infection due to L. canicola appears to produce a higher titer than L. icterohaemorrhagiae. This high titer was observed in the serological results in the 500 normal dogs tested. It was further substantiated in the results of the 30 active infections of the disease. In Experiment 2, only 2 (15.4%) of the sera demonstrated a 1:1,000 or greater specific titer to L. icterohaemorrhagiae. Whereas, 35 (26.7%) of the sera gave a 1:1,000 or greater specific titer with L. canicola.

Non or paraspecific reactions, especially in the higher dilutions, occurred with sera giving a specific titer to both antigens. As is

summarized in Table 7 and 8, 75 (57.25%) of the positive L. canicola samples and 5 (38.40%) of the L. icterohaemorrhagiae positive samples demonstrated paraspecific titers. The L. canicola-positive sera demonstrate a higher percentage of paraspecific reactions. Regardless of specific antigen, paraspecific reactions occurred more commonly in sera demonstrating a high specific titer.

TABLE 4

The reactions of 148 serologically positive canine sera samples

Specific Positive Titers	Number of Samples	Per cent of Reactors	Per cent of Total
L. icterohaemorrhagiae	13	8.8	2.6
L. canicola	131	88.5	26.2
L. icterohaemorrhagiae and *L. canicola* equal	4	2.7	0.8
Total	148		29.6

TABLE 5

Age incidence of latent canine leptospirosis

of the 500 dogs tested

Age Group	Number Tested	Number Positive	Per cent Positive	Per cent of Age Group Infected
1 mo. to 1 yr.	97	26	17.50	26.90
1 yr. through 3 yrs.	221	38	25.70	17.40
4 yrs. through 7 yrs.	134	57	38.55	42.60
8 yrs. through 12 yrs.	47	27	18.25	57.40

TABLE 6

Sex incidence of latent canine leptospirosis in the Lansing, Michigan, area

Sex	No. Tested	Per cent of Total	No. Reactors	Per cent Reactors	No. of Sex Reacting *L. canicola*	Per cent of Sex Reacting *L. canicola*	No. of Sex Reacting *L. icterohaemorrhagiae*	Per cent of Sex Reacting *L. icterohaemorrhagiae*	No. of Sex Reacting Equal Titre	Per cent of Sex Reacting Equal Titre
Males	279	55.80	104	70.40	91	87.50	10	9.62	3	2.88
Females	221	44.20	44	29.60	40	90.00	3	6.82	1	2.28

TABLE 7

Titer of specific and paraspecific reactions

Infection with L. canicola

Specific *L. canicola* titer	Number	Paraspecific *L. icterohaemorrhagiae* titer					
		Neg.	10	30	100	300	1,000
10	8	8	0	0	0	0	0
30	14	10	4	0	0	0	0
100	39	19	8	12	0	0	0
300	35	13	5	7	8	0	0
1,000	21	5	2	5	5	4	0
3,000	8	1	0	1	3	4	0
10,000	2	0	0	0	0	2	0
30,000	4	0	0	0	1	2	1
Total	131*	56	19	25	17	12	1

*4 sera, titers equal for both strains, not included

TABLE 8

Titer of specific and paraspecific reactions

Infection with L. icterohaemorrhagiae

Specific L. icterohaemorrhagiae titer	Number	Paraspecific L. canicola titer					
		Neg.	10	30	100	300	1,000
10	0	0	0	0	0	0	0
30	1	1	0	0	0	0	0
100	6	4	2	0	0	0	0
300	4	3	0	0	1	0	0
1,000	2	0	0	0	1	1	0
3,000	0	0	0	0	0	0	0
10,000	0	0	0	0	0	0	0
30,000	0	0	0	0	0	0	0
Total	13	8	2	0	2	2	0

*4 sera, titers equal for both strains, not included

DISCUSSION

Canine leptospirosis may be caused by either L. canicola or L. icterohaemorrhagiae. The two species are culturally and morphologically identical (Morton, 1943), although they may be differentiated serologically. The incubation period for the disease varies from 1-2 weeks with a mortality rate of 0-25 per cent. The organisms are present in the blood from the 1st to the 6-8th day after the appearance of symptoms or during the febrile period. After this time, they disappear from the blood and localize in the kidneys, resulting in leptospiruria. On the 9-10th day the antibody titer begins to rise, reaching a maximum within a few weeks, and is maintained for a period of several months. The drop to lower titer levels is gradual, and some titer may persist for as long as 2-3 years.

Clinical observations of the 30 proven cases of canine leptospirosis due to L. canicola were similar to those observed by Jungherr (1937), Meyer (1939a), and Elander and Perry (1940). Two distinct clinical types were observed, the icteric and the hemorrhagic. Icterus occurred in 6 of the 30 cases. Depression, high temperature, vomiting, posterior weakness, refusal to eat, and congested conjunctivae occurred in the majority of the cases.

Dogs may become infected with L. icterohaemorrhagiae through contact with infected rat urine. Rats from various parts of the world have been examined and as high as 45 per cent have demonstrated the organisms in the kidney tubules (Ido and associates, 1917; Noguchi, 1917; Jobling and Eggstein, 1917; Langworth and Moore, 1927; Walch-Sorgdrager, 1939; Meyer and coworkers, 1939, and Monlux, 1948).

Canine L. canicola infections are maintained by simple contact from dog to dog. Natural infections have not been reported in other animals except man. The elimination of the organisms in the urine is responsible for the spread of the disease. The organisms are capable of living outside of the body for varying periods of time (Davidson and coworkers, 1934). Meyer and coworkers (1938) and Walch-Sorgdrager (1939) have not encountered L. canicola in the rat population. It is believed justified to conclude that the rat is not the natural host for L. canicola and need not be considered in the epizootology of the disease.

The results of Experiment 1 indicate that none of the laboratory diagnostic procedures are sufficiently reliable at all times. It is impossible for a veterinarian to diagnose canine leptospirosis, except clinically, when the animal is first present. Only 10 per cent and 23.33 per cent of the stained and darkfield blood sediment preparations, respectively, demonstrated Leptospira. Their efficiency as diagnostic procedures is apparent. False positives in stained smears are practically nonexistent, although caution should be exercised in the interpretation of darkfield preparation. Schultz (1923) and Hall (1925) observed motile filamentous elements in the blood, highly suggestive of living organisms. The "pseudospirochetes" have been observed in the blood of normal and diseased individuals. The novice may be misled by their presence. If one thoroughly acquaints himself with the morphology and motility of these "pseudospirochetes" and Leptospira, fewer false positives will be recorded.

The cultural examinations of blood yielded isolations in 6 (20%) of the animals examined. Had cultural examinations of blood been made

on or before the 4-5th day of illness, more satisfactory results would have been obtained. This confirms the results of all workers in their isolation attempts from humans infected with Leptospira (Davidson, et al., 1934; Smith and Tulloch, 1937; Packchanian, 1941; Bertucci, 1945).

Table 2 indicates that the accuracy of the agglutination test increases as the disease progresses. The results reported here (Table 2) indicate that at the end of 12-15 days and 21-30 days of illness it is approximately 50 and 100 per cent accurate, respectively. All workers (Bertucci, 1945) agree that strong false positive reactions are non-existent. If serial agglutination tests are conducted, the exact status of the titer may be determined. A 4-plus agglutination reaction in the 1:100 serum dilution was employed as the diagnostic criterion. Experiment 2 indicates that at least 29 per cent of the animals in this area appear to demonstrate some titer to one or both antigens.

Although the agglutination test is a rapid, accurate, laboratory diagnostic procedure, it must be emphasized that its usefulness is limited. At the time an accurate diagnosis is obtained by this method, the animal is either dead or on its way to recovery. Hence, the immediate instigation of specific therapeutic measures is not possible. It would appear, however, that it is fully reliable in retrospective diagnosis as the results of Experiment 1 and 2 indicate.

The incidence of latent canine leptospirosis has been summarized from comparable reports in Table 9. The world incidence is 0-45 per cent, the highest incidence occurring in Holland, where perhaps the most extensive studies on both human and canine leptospirosis have been conducted (references previously cited). In the United States the

range is 0-33.33 per cent, the highest occurring in Pennsylvania and none reported from Alabama. The results reported here correlate closely with other reports from this country, although the incidence of L. icterohaemorrhagiae canine infection is slightly lower.

There appears to be an explanation for the difference between the results obtained by Meyer (1939) and the results reported here. The 10 samples examined by Meyer were obtained from the Detroit area, from which Molner and Kasper (1938), Molner and Meyer (1940), Molner, Meyer, and Raskin (1948) reported 75 human cases of Weil's disease. In addition, Meyer and associates (1939) reported that 16 per cent of the rats in that area demonstrated renal leptospiral infections. Thus, because of environmental conditions which exist in the Detroit area, the incidence of L. icterohaemorrhagiae infections is higher than in the Lansing area. It should be mentioned, however, that the canine sera samples submitted to the author for examination from the Detroit area, indicate that L. canicola infections are also present.

Evidence supported by the findings in Experiment 2 (Table 4), indicate that many of the atypical canine illnesses may be subclinical or latent leptospirosis. Leptospiruria is known to occur in those dogs infected and their role as spreaders of the disease to other dogs and man should be emphasized.

TABLE 9

Incidence of latent canine leptospirosis

State or Country	Positive *L. canicola*		Positive *L. icterohaemorrhagiae*		Number Dogs Examined
	No.	%	No.	%	
Holland[3]	25	32.0	10	13.0	76
Holland[3]	10	20.0	4	8.0	50
Germany[3]	6	12.0	3	6.0	48
Germany[3]	3	1.5	1	0.5	200
Denmark[3]	1	1.9	18	34.0	53
Alabama[3]	0	0.0	0	0.0	21
California[3]	20	26.0	0	0.0	75
New York[3]	10	9.0	3	2.7	111
Pennsylvania[1]	11	22.0	3	6.0	50
Pennsylvania[2]	26	24.8	9	8.5	105*
Michigan[3]	0	0.0	10	76.0	13
Michigan[4]	131	26.8	13	2.6	500**

[1]Dogs from the urban area - reported by Raven (1941)
[2]Dogs from the rural area - reported by Raven (1941)
[3]Reported by K. F. Meyer (1939a)
[4]Reported by author
*Five sera demonstrated an equal titer for both antigens.
**Four sera demonstrated an equal titer for both antigens.

SUMMARY

1. Staining, cultural, and serological procedures were conducted on blood and urine sediments of 30 naturally-infected dogs to determine their value as laboratory diagnostic procedures of infection.
2. The staining and cultural procedures are considered unreliable as diagnostic procedures, although the agglutination test was approximately 50 per cent and 100 per cent accurate after the 2nd and 3-4th week of illness, respectively.
3. The sera from 500 normal dogs were examined by the agglutination test in retrospective diagnosis. Twenty-nine and six-tenths per cent of the dogs demonstrated some titer to either L. canicola or L. icterohaemorrhagiae antigens, or both. Twenty-six and two-tenths per cent of these were positive to L. canicola, two and six-tenths per cent were positive to L. icterohaemorrhagiae and eight-tenths per cent demonstrated an equal positive titer to both.
4. Male to female incidence was approximately 2:1. The dogs were arbritarily divided into age groups, the incidence of infection increasing with age.
5. The results of Experiment 2 indicate that nearly one-third of the dogs in this area are possible reservoirs for Leptospira, thus serving to disseminate the disease to other dogs and man.

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