

Clinical and pathologic comparison of acute leptospirosis in dogs caused by two strains of *Leptospira kirschneri* serovar grippotyphosa

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Objective—To develop a method for inducing acute leptospirosis in dogs.

Animals—31 nine-week-old female Beagles.

Procedure—Beagles were randomly assigned to 2 inoculation groups or a control group. Dogs were inoculated on 3 successive days by conjunctival instillation of 5×10^7 cells of *Leptospira kirschneri* serovar grippotyphosa strain 82 (12 dogs) or strain RM 52 (14 dogs). Control dogs ($n = 5$) were similarly inoculated with sterile leptospiral culture media. Clinical signs, clinicopathologic variables, anti-leptospiral antibody titers, and evidence of leptospires in tissues and body fluids were evaluated. Dogs were euthanatized and necropsied on days 7, 14, 22, or 28 after inoculation or as required because of severe illness.

Results—Clinical signs in infected dogs included conjunctivitis, lethargy, diarrhea, dehydration, vomiting, and icterus. Consistent clinicopathologic alterations included azotemia, hyperphosphatemia, increased anion gap, hyperbilirubinemia, and an increase in alkaline phosphatase activity. Leptospires were cultured from the kidneys (11/12), urine (6/9), aqueous humor (9/12), blood (12/12), and liver (12/12) of dogs inoculated with strain 82. Only 3 of 14 dogs became infected after inoculation with strain RM 52. Histopathologic lesions in infected dogs included interstitial nephritis, renal tubular degeneration and necrosis, pulmonary hemorrhage, and hepatic edema and perivascularitis.

Conclusions and Clinical Relevance—Conjunctival exposure to *L kirschneri* serovar grippotyphosa strain 82 resulted in acute leptospirosis in all inoculated dogs, but only 3 of 14 dogs inoculated with strain RM 52 became infected. This method of infection by serovar grippotyphosa can be used to study the pathogenesis and prevention of leptospirosis in dogs. (*Am J Vet Res* 2004;65:1100–1107)

Leptospirosis, one of the most widespread zoonotic diseases, is a reemerging disease of dogs.¹ The diagnosis of leptospirosis in dogs in the United States and Canada increased substantially between 1983 and

1998.² In the United States, leptospirosis in dogs has traditionally been associated with *Leptospira interrogans* serovars canicola and icterohaemorrhagiae. Experimentally induced disease caused by serovars canicola and icterohaemorrhagiae has been described,³⁻¹⁰ and the use of vaccines containing these serovars has markedly reduced the incidence of disease attributable to them.^{3,4} Vaccines have been available to protect against only those serovars; however, during the past 10 years, there has been an increase in the number of dogs with leptospirosis from which clinicians have isolated or detected serologic evidence to support the involvement of *L kirschneri* serovar grippotyphosa, *L interrogans* serovar bratislava, and *L interrogans* serovar pomona.¹⁻³ Features of disease caused by these serovars (eg, time course for the disease, lesions, and biochemical abnormalities that may be serovar-specific) have not been clearly identified because of the paucity of experimental studies and the inability to determine the time of exposure in many clinical cases.

Reports^{5,6} on experimental investigation of the course of disease, lesions, and confirmation of infection by serovars grippotyphosa, bratislava, and pomona in dogs are sparse despite the emergence of these serovars as important causes of renal and hepatic disease of dogs in North America.^{1,3,7,8} Better understanding of the progression of clinical signs and clinicopathologic abnormalities in the course of disease is needed for these serovars. The purpose of the study reported here was to inoculate dogs with *L kirschneri* serovar grippotyphosa in controlled conditions and document clinical signs, changes in results of CBC counts and biochemical analyses, and gross and histologic lesions.

Materials and Methods

Dogs—Thirty-one 9-week-old purpose-bred female Beagle pups that lacked detectable leptospiral antibodies to serovar grippotyphosa, as determined by use of the microscopic agglutination test (MAT),⁹ were used in the study. Dogs were housed in pairs in temperature-controlled rooms

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at the Iowa State University Biosecurity Level-2 Animal Holding Facility. Dogs were allocated to 3 groups. Separate rooms were used for control dogs (n = 5), dogs inoculated with serovar grippotyphosa strain RM 52 (14), and dogs inoculated with serovar grippotyphosa strain 82 (12). Dogs were fed a high-quality commercial dry ration. Food and water were available at all times and freshened twice daily. During the acclimation period, CBCs, serum biochemical analyses, and urinalyses were performed on each dog; no abnormalities were detected. Twice daily, the dogs were observed for clinical signs of disease and body temperatures were recorded. All animal experiments were approved by the Iowa State University Committee on Animal Care.

Leptospire—Two strains of *L. kirschneri* serovar grippotyphosa were used. Serovar grippotyphosa strain RM 52 was isolated at the National Animal Disease Center from material submitted during an outbreak of abortions in pigs in 1983.^{10,11} Inoculum was prepared from cryopreserved leptospire. To prevent loss of virulence through adaptation to culture conditions, leptospire were passaged through hamsters¹² and reisolated. Hamsters were housed at the National Animal Disease Center and received IP inoculations consisting of 10⁶ organisms. Liver homogenate obtained from a hamster with clinical signs of disease was cultured in 80-40 semisolid media to provide the inoculum used in the study reported here.

Strain 82 was isolated at the National Animal Disease Center from the urine of a dog examined in 1992 because of polyuria and polydipsia. That dog had mild azotemia, hypercalcemia, isostenuria (specific gravity, 1.008), and a titer of 1:1,600 for serovar grippotyphosa. In addition, urine samples obtained from that dog contained leptospire, as determined by use of immunofluorescence testing. This isolate was also passaged through hamsters. Hamsters received IP inoculations consisting of 10⁶ organisms. Liver homogenate obtained from a hamster with clinical signs of disease was cultured in 80-40 semisolid media to provide the inoculum used in the study reported here.

Experimental design—All inoculations were administered via conjunctival instillation. Inoculum was prepared as described elsewhere.¹⁰ Each inoculum consisted of 5 × 10⁷ leptospire in 500 µL of 80-40 semisolid leptospiral culture medium¹³; 250 µL was placed on the corneal surface of each eye of each dog and allowed to flow onto the conjunctiva. This procedure was repeated once daily for 3 consecutive days. The first day of inoculation was designated as day 0. Twelve dogs were inoculated with strain 82, and 14 dogs were inoculated with strain RM 52. The 5 control dogs were inoculated with an equal volume of sterile culture media.

Sample collection—Blood samples for CBCs and serum biochemical analysis that included concentrations of sodium, potassium, chloride, total carbon dioxide (TCO₂), calcium, phosphorus, BUN, creatinine, glucose, total protein, albumin, direct bilirubin, and total bilirubin; activities of alanine transaminase (ALT), alkaline phosphatase (ALP), γ -glutamyltransferase (GGT), and creatine kinase; and measurement of the anion gap were obtained before inoculation (baseline) and immediately before dogs were euthanatized on days 7, 14, 22, or 28 after inoculation. When dogs died or were euthanatized on days other than these, appropriate samples were collected at that time. Values of serum biochemical analysis of samples obtained at the time of necropsy were compared with baseline values by use of a paired t test.

Necropsy—Necropsies were scheduled for days 7, 14, 22, and 28 after inoculation. Dogs were euthanatized immediately and necropsied when examinations revealed clinical signs of severe disease (> 5% dehydrated; had epistaxis; were

anorectic, anuric, or febrile for > 24 hours; or had episodes of vomiting or diarrhea that did not resolve within 24 hours). Before being euthanatized, dogs were injected with furosemide^a to increase urine production and enhance the probability that investigators would be able to collect a urine sample for leptospiral culture.¹⁴ Dogs were euthanatized by administration of an overdose of sodium pentobarbital.^b Samples of the liver, kidneys, and urine were collected aseptically for leptospiral culture and immunofluorescence testing. Samples of the kidneys, liver, lungs, hepatic lymph nodes, adrenal glands, spleen, heart, tonsils, mesenteric lymph nodes, pancreas, stomach, jejunum, vulva, bladder, bone marrow, iliac lymph nodes, colon, ileum, and cecum were collected and placed in neutral-buffered 10% formalin. Samples of the eyes and conjunctiva were collected and placed in Bouin's solution.

Histologic examination—Samples remained in formalin fixative for 24 hours and were then processed for histologic evaluation by use of standard paraffin-embedding techniques. Tissues were sectioned at a thickness of 5 µm, except for the kidneys, lymph nodes, and adrenal glands, which were sectioned at a thickness of 3 µm. All sections were stained with H&E.

Histologic grading of selected lesions of the kidneys, liver, and pancreas was accomplished by use of a 4-point scale (no change from control dogs, mild lesions, moderate lesions, and severe lesions). Scores for interstitial nephritis, tubular mineralization, and hepatic vasculitis were assigned as follows: -, no change; +, 1 to 3 foci/section examined; ++, 4 to 6 foci/section examined; and +++, > 6 foci/section examined. Scores for tubular degeneration were assigned as follows: -, no change; +, scattered cells with pyknotic nuclei or loss of polarity; ++, aforementioned changes were evident in larger sections of a tubule; and +++, multiple tubules in an area affected. Scores for tubular dilatation were assigned as follows: -, no change; +, scattered tubular lumens markedly widened and lined by a thin layer of epithelium; ++, multiple tubules affected; and +++, cortex diffusely affected. Scores for dissociation of hepatocytes from laminae (individualization) and necrosis were assigned as follows: -, no change; +, hepatocyte cell margins were more distinct than cell margins for control dogs; ++, small pockets of hepatocytes without connections to adjacent cells; and +++, aforementioned changes were evident in large sheets of cells. Scores for interstitial pancreatitis were assigned as follows: -, no change; +, small numbers of inflammatory cells between lobules; ++, infiltration of the lobules by inflammatory cells; and +++, diffuse inflammation throughout the pancreas.

Leptospiral culture—Blood samples collected 6, 8, 10, 12, 14, 22, and 28 days after inoculation were cultured for leptospire. Samples of the liver and kidneys (approx 1 g), aqueous humor (1 mL), and urine (1 mL) were obtained during necropsy and cultured for leptospire. Three media were used to increase the likelihood of isolating leptospire. Tween 80-Tween 40-lactalbumin hydrolysate was used as described elsewhere,¹³ with slight modifications (5-fluorouracil^{15c} was used at a rate of 100 mg/mL, and nalidixic acid was not used). Ellinghausen-McCullough-Johnson-Harris semisolid media was used with the addition of 1% bovine serum albumin, 5-fluorouracil (100 µg/mL), and 1% rabbit serum, as described elsewhere.¹⁶ A commercially available polysorbate leptospiral medium^d was used with the addition of purified agar^e and 5-fluorouracil (100 µg/mL). Cultures were incubated at 29°C for 6 months. Darkfield examination was performed 1, 2, 4, 6, and 8 weeks and 3, 4, 5, and 6 months after start of culturing. When cultures became contaminated, approximately 1 mL of medium was filtered through a 0.22-µm filter and inoculated into new medium. Representative

isolates were identified by use of restriction endonuclease analysis of chromosomal DNA.¹⁷

Serologic testing—Blood samples for use in serologic testing were collected before and 7, 14, 22, and 28 days after inoculation from 6 dogs. Blood samples also were collected before necropsy from 7 dogs inoculated with strain 82, 9 dogs inoculated with strain RM 52, and 4 control dogs. Agglutinating antibodies to serovar grippotyphosa were detected by use of the MAT.⁹

Immunofluorescence testing—Samples of the kidneys, liver, urine, aqueous humor, and liver were stained with fluorescein-labeled rabbit anti-leptospiral conjugate, as described elsewhere.¹⁸ Leptospire were identified on the basis of typical size, morphology, and fluorescence when examined by fluorescence incident light fluorescence microscopy.

Immunohistochemical analysis—After infection was confirmed on the basis of positive results for leptospiral culture or immunofluorescence testing, immunohistochemical staining was performed on samples obtained from a subset of dogs to determine the patterns of antigen distribution. A monoclonal mouse antibody with reactivity specific for serovar grippotyphosa was used as described elsewhere,^{10,19} with slight modifications. Briefly, tissues were sectioned (thickness of 5 µm). Sections were placed on positively charged slides and deparaffinized with xylene and ethanol, in accordance with standard procedures. Slide-mounted tissue sections were rinsed in potassium PBS solution (KPBSS; 0.15M NaCl, 0.034M K₂HPO₄, and 0.017M KH₂PO₄ [pH, 7.4]), and antigen retrieval was performed by incubating slides in 0.1% trypsin and 0.1% CaCl₂ for 7 minutes at 37°C. Slides were then rinsed twice in KPBSS, and endogenous peroxidase activity was eliminated by the addition of 0.3% hydrogen peroxide solution in KPBSS and incubation for 30 minutes. Blocking solution (KPBSS with 1% bovine serum albumin,^f 0.4% Triton X-100,^g and 1.5% normal blocking serum^h) was added, and sections were incubated for 2 hours. Sections were incubated overnight in primary antiserum at room temperature (24°C). The following day, tissue sections were rinsed in KPBSS with 0.2% Triton X-100 and incubated in an appropriate biotinylated secondary antibody for 2 hours at room temperature. Sections were then rinsed, horseradish peroxidase-avidin-biotin complexⁱ was added, and sections were incubated for 1 hour at room temperature. Enzyme reactions were accomplished by use of a substrate kitⁱ that caused development of a red reaction. The reaction was terminated in successive rinses of saline (0.9% NaCl) solution. Slides were counterstained with hematoxylin and dehydrated by immersion in a series of graded alcohol solutions. Finally, coverslips were applied.

Results

Clinical signs and leptospiral culture—Dogs were considered infected when leptospire were detected in

tissues or body fluids by use of leptospiral culture, immunofluorescence testing, or immunohistochemical analysis. Results of leptospiral culture and immunofluorescence testing were negative in all 5 control dogs (Table 1). Serovar grippotyphosa was detected in 3 of 14 dogs inoculated with strain RM 52 and all 12 dogs inoculated with strain 82. Mild redness of the sclera and conjunctiva was evident in all leptospire-inoculated dogs on day 1 (ie, second day of inoculation). On day 5, inoculated dogs were lethargic, and 1 dog from each of the serovar grippotyphosa-inoculated groups had severe redness of the sclera and conjunctivitis with thick ocular discharge. On day 8, 2 dogs inoculated with strain 82 were febrile (40.1° and 40°C, respectively). A third dog was febrile (39.4°C) on day 9.

Control dogs were euthanatized on days 7 (n = 1), 14 (1), 22 (1), or 28 (2). Two dogs inoculated with strain 82 died (1 on day 11 and 1 on day 12), and 10 inoculated dogs were euthanatized prior to their scheduled date. Dogs were euthanatized because of lethargy, dehydration, subnormal temperature, conjunctivitis, and icterus. Dogs were euthanatized on days 9 (1 dog inoculated with strain 82), 10 (1 dog inoculated with strain 82), 11 (1 dog inoculated with strain 82), 12 (4 dogs inoculated with strain 82 and 2 dogs inoculated with strain RM 52), or 13 (1 dog inoculated with strain RM 52). The 3 remaining dogs inoculated with strain 82 were euthanatized on day 7, whereas remaining dogs inoculated with strain RM 52 were euthanatized on days 7 (n = 3), 14 (2), 22 (3), or 28 (3).

Clinical signs before dogs died or were euthanatized were similar between dogs inoculated with strains RM 52 and 82, but a greater percentage of dogs inoculated with strain 82 had clinical signs. Clinical assessments revealed that inoculated dogs were affected by dehydration (2 dogs for strain RM 52 and 7 dogs for strain 82), icterus (2 dogs for strain RM 52 and 9 dogs for strain 82), lethargy (2 dogs for strain RM 52 and 6 dogs for strain 82), coughing (1 dog for strain RM 52 and 1 dog for strain 82), diarrhea (3 dogs for strain RM 52 and 6 dogs for strain 82), and vomiting (2 dogs for strain RM 52 and 5 dogs for strain 82).

Necropsy—Gross findings during initial examination included icterus, conjunctivitis, and ocular discharge. Lesions were found in the kidneys, liver, and lungs (Table 2). Lesions detected during necropsy were similar for all dogs euthanatized on days 9 through 12 and included icterus of the sclera, gingiva, and subcutaneous tissues; a friable, pale liver with enhanced lob-

Table 1—Results of leptospiral culture and immunofluorescence testing of various samples obtained from control dogs, dogs inoculated with *Leptospira kirschneri* serovar grippotyphosa strain 82, and dogs inoculated with *L kirschneri* serovar grippotyphosa strain RM 52.

Group	Leptospiral culture					Immunofluorescence testing		
	Blood	Kidneys	Liver	Urine	Aqueous humor	Kidneys	Liver	Urine
Control	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Strain 82	12/12	11/12	12/12	6/9*	9/12	10/12	8/12	5/9*
RM 52	3/14	3/14	3/14	1/14	1/14	2/14	2/14	0/14

Values reported are number of positive results obtained per number of dogs tested.
*Urine samples were not available from 3 dogs.

Table 2—Major lesions detected in the kidneys, liver, and pancreas for control dogs, dogs inoculated with *L. kirschneri* serovar grippotyphosa strain 82, and dogs inoculated with *L. kirschneri* serovar grippotyphosa strain RM 52.

Group	Day*	Kidneys				Liver			Interstitial pancreatitis
		Interstitial nephritis	Tubular mineralization	Tubular degeneration	Tubular dilatation	Vasculitis	Dissociation	Necrosis	
Control (n)									
1	7	1-	1-	1-	1-	1-	1-	1-	1-
1	14	1-	1-	1-	1-	1-	1-	1-	1-
1	22	1-	1-	1-	1-	1-	1-	1-	1-
2	28	2-	2-	2-	2-	2-	2-	2-	2-
Strain 82 (n)									
3	7	3+	3-	2-, 1+	3-	1++, 2+	3-	2-, 1+	1-, 1+, 1++
1	9	1++	1-	1++	1-	1+	1+++	1+	1+
1	10	1+++	1++	1+++	1-	1++	1++	1+	1+
2	11	2+++	1-, 1+	1+++†	1-†	2+	1+, 1++	2+	1-, 1+
5	12	3+++, 2+++	2-, 1+, 1++, 1+++	2+, 2+++†	1+, 3+++†	1-, 3+, 1++	2-, 2+, 1++	1-, 3+, 1++	3-, 1+†
Strain RM 52 (n)									
3	7	3-	3-	3-	3-	3-	3-	3-	3-
2	12	2+++	2-	1+, 1++	1+, 1++	1++, 1+++	1+, 1++	1-, 1++	2-
1	13	1+++	1+	1+	1++	1-	1-	1+	1+
2	14	2-	2-	2-	2-	2-	2-	2-	2-
3	22	3-	2-, 1++	3-	3-	1-, 2+	3-	3-	3-
3	28	3-	3-	3-	3-	3-	3-	3-	3-

Values reported are the number of dogs with a particular score for that variable for that time period.

*Represents the day after inoculation on which the dogs were necropsied and specimens obtained; day 0 was the first day of a 3-day inoculation procedure. †Includes data from 1 dog that died and had autolytic tissues that precluded assessment of lesions. ‡Includes 1 sample that was not examined.

Histologic grading of selected lesions of the kidneys, liver, and pancreas was accomplished by use of a 4-point scale (no change from control dogs, mild lesions, moderate lesions, and severe lesions). Scores for interstitial nephritis, tubular mineralization, and hepatic vasculitis were assigned as follows: -, no change; +, 1 to 3 foci/section examined; ++, 4 to 6 foci/section examined; and +++, > 6 foci/section examined. Scores for tubular degeneration were assigned as follows: -, no change; +, scattered cells with pyknotic nuclei or loss of polarity; ++, aforementioned changes were evident in larger sections of a tubule; and +++, multiple tubules in an area affected. Scores for tubular dilatation were assigned as follows: -, no change; +, scattered tubular lumens markedly widened and lined by a thin layer of epithelium; ++, multiple tubules affected; and +++, cortex diffusely affected. Scores for dissociation of hepatocytes from laminae (individualization) and necrosis were assigned as follows: -, no change; +, hepatocyte cell margins were more distinct than cell margins for control dogs; ++, small pockets of hepatocytes without connections to adjacent cells; and +++, aforementioned changes were evident in large sheets of cells. Scores for interstitial pancreatitis were assigned as follows: -, no change; +, small numbers of inflammatory cells between lobules; ++, infiltration of the lobules by inflammatory cells; and +++, diffuse inflammation throughout the pancreas.

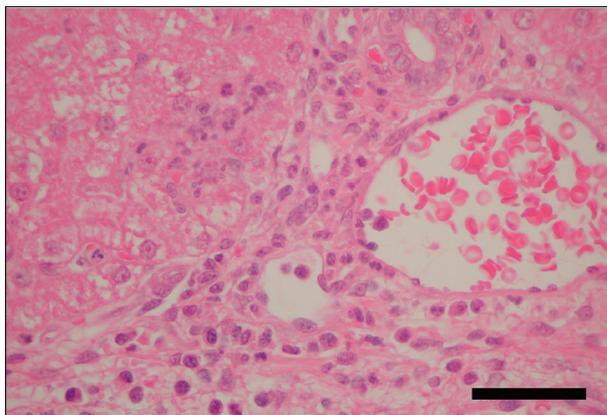


Figure 1—Photomicrograph of a section of liver obtained from a dog 7 days after inoculation with *Leptospira kirschneri* serovar grippotyphosa strain 82. The portal area is infiltrated by small numbers of lymphocytes and plasma cells and a few neutrophils that surround the hepatic vein, lymphatic duct, and bile duct. H&E stain; bar = 40 μ m.

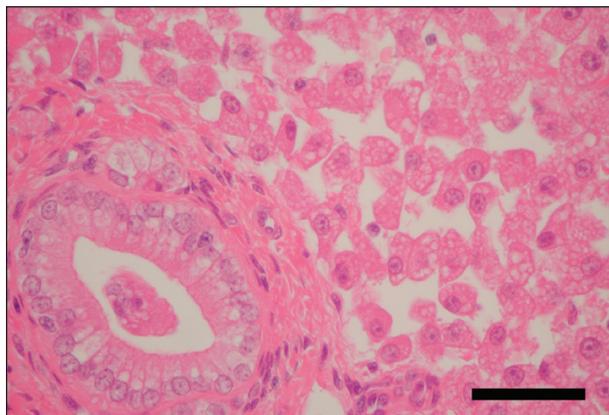


Figure 2—Photomicrograph of a section of liver obtained from a dog 9 days after inoculation with serovar grippotyphosa strain 82. The hepatocytes are dissociated from hepatic laminae and separated by edema, and the cytoplasm contains multiple small, clear vacuoles (ie, lipid). H&E stain; bar = 40 μ m.

ular pattern; subcutaneous and retroperitoneal edema; multifocal petechiae in the lungs; prominent red lymph nodes (hepatic or internal iliac lymph nodes); and enlarged kidneys with perirenal edema. Typically, stomach and intestinal contents were scant. One dog inoculated with strain 82 that had been vomiting had an ileocecal intussusception (10 cm) with devitalized ileum.

Histologic examination and immunohistochemical analysis—Lesions were identified in the liver, kid-

neys, and pancreas (Table 2). Lesions in the liver included mixed perivascular infiltrates of neutrophils, lymphocytes, and plasma cells on days 7 to 12 and dissociation of hepatocytes from hepatic laminae and piecemeal hepatic necrosis with multifocal plugs of bile pigment within bile canaliculi on days 9 to 12 (Figures 1 and 2). Increased numbers of mitotic figures were evident in hepatocytes of infected dogs on day 12 (Figure 3). Mitotic figures were detected at a rate of 1 to 3/HPF (ie, 400X magnification) examined

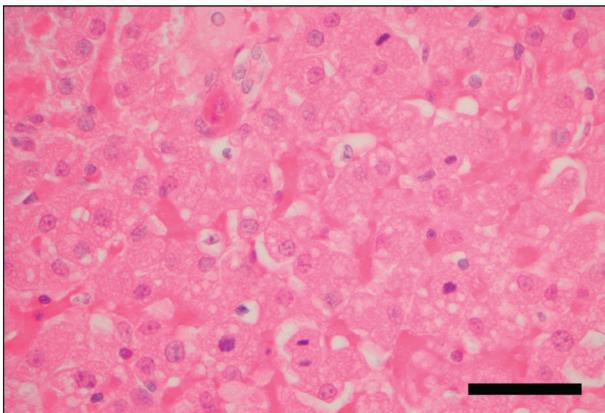


Figure 3—Photomicrograph of a section of liver obtained from a dog 12 days after inoculation with serovar grippotyphosa strain 82. Notice that mitotic figures are common. Also, the hepatocytes contain lipid-filled vacuoles. H&E stain; bar = 40 μ m.

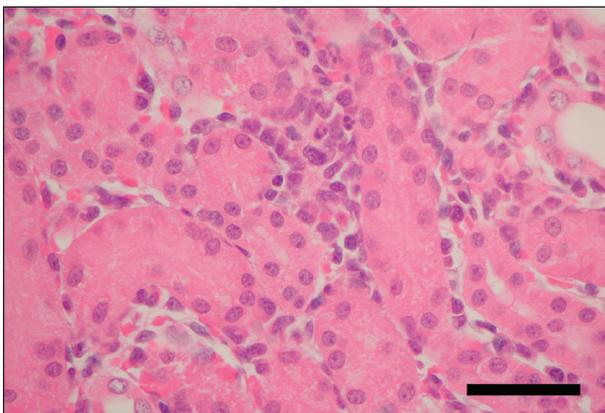


Figure 4—Photomicrograph of a section of the kidneys obtained from the dog in Figure 1. There is slight expansion of the interstitium by infiltrates of lymphocytes and plasma cells and a few neutrophils. H&E stain; bar = 40 μ m.

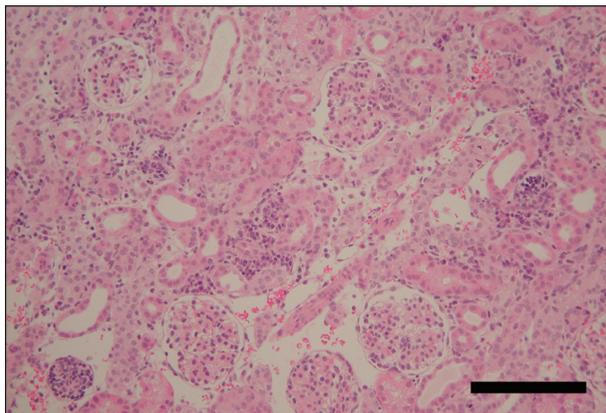


Figure 5—Photomicrograph of a section of the kidneys obtained from a dog 12 days after inoculation with serovar grippotyphosa strain 82. Notice that the interstitium is expanded by edema, free erythrocytes, and inflammatory infiltrates. In addition, there are multifocal, dilated, proximal convoluted tubules with flattened epithelium. H&E stain; bar = 140 μ m.

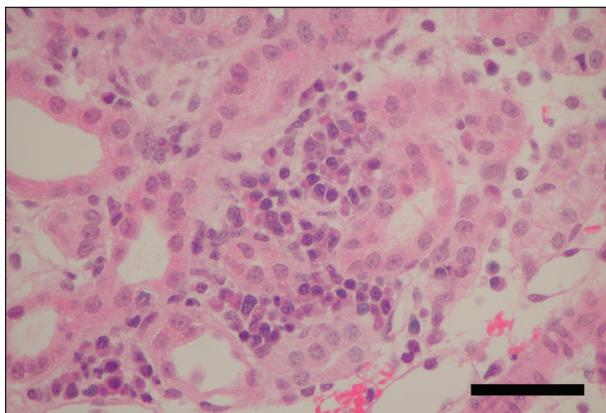


Figure 6—A higher magnification view of the photomicrograph of Figure 5. The interstitial infiltrate is composed primarily of plasma cells and lymphocytes. H&E stain; bar = 40 μ m.

in inoculated dogs but were rare ($< 1/5$ HPFs) in control dogs. Prominent lesions in the kidneys included interstitial nephritis, mineralization, tubular dilatation, and tubular degeneration (Figures 4–6). Infiltrates of neutrophils between lobules of the pancreas were evident in 6 dogs (Figure 7). Immunoreactivity to serovar grippotyphosa lipopolysaccharide was detected in the lumens of the proximal convoluted tubules and within interstitial vessels. Sites of immunoreactivity were small and multifocal with a granular to globular appearance.

Clinicopathologic examinations—Consistent clinicopathologic alterations at necropsy in dogs inoculated with strain 82 were azotemia, hyperphosphatemia, an increase in anion gap, hyperbilirubinemia, and an increase in ALP activity (Table 3). When necropsy data for BUN, creatinine, phosphorus, TCO₂, and total bilirubin concentrations; anion gap; and ALT, ALP, and GGT activities in dogs inoculated with strain 82 were compared with baseline values for that group by use of the paired *t* test, significant ($P < 0.001$) differences were detected. Results of a CBC for dogs inoculated with strain 82 included leukocytosis with mature neutrophilia ($P = 0.003$). The 2 dogs inoculat-

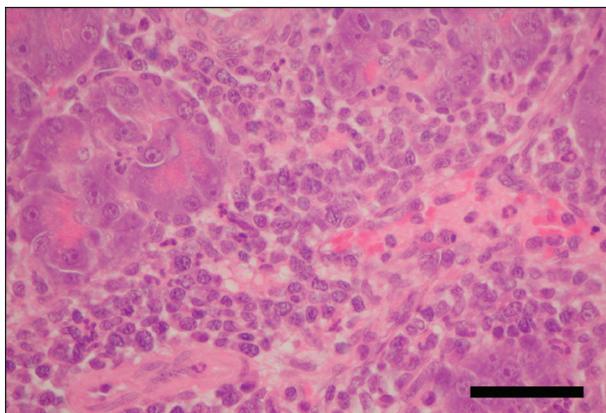


Figure 7—Photomicrograph of a section of pancreas from the dog in Figure 1. Notice that the interlobular interstitium is infiltrated by moderate numbers of a mixed population of lymphocytes, plasma cells, neutrophils, and macrophages. H&E stain; bar = 40 μ m.

ed with strain RM 52 that developed infection and for which samples were available for analysis had similar CBC and serum biochemical abnormalities that included neutrophilia, marked hyperkalemia, relative hypercalcemia, hypoalbuminemia, hyperphosphatemia,

Table 3—Mean \pm SD values for selected clinicopathologic variables of dogs before (baseline) and 7 to 28 days after inoculation with *L. kirschneri* serovar grippotyphosa strain 82.

Selected variable	Baseline	After inoculation	Reference range
BUN (mg/dL)	15 \pm 5*	220 \pm 52	10–30
Creatinine (mg/dL)	0.3 \pm 0.1*	4.6 \pm 1.5	0.1–1.2
Phosphorus (mg/dL)	8.3 \pm 0.7*	21.9 \pm 5.8	3.2–6.2
Anion gap	19.4 \pm 1.5*	37.1 \pm 7.5	11–20
Total CO ₂ (mEq/L)	23.9 \pm 1.7*	14.9 \pm 2.8	17–24
ALT (IU/L)	25.0 \pm 4.4*	71.4 \pm 18.4	24–105
ALP (IU/L)	173 \pm 77*	1,269 \pm 584	20–115
GGT (IU/L)	5.8 \pm 5.5*	15.2 \pm 3.7	1–10
Total bilirubin (mg/dL)	0.2 \pm 0.1*	4.8 \pm 1.7	0.1–0.6
WBC ($\times 10^6/\mu\text{L}$)	15.6 \pm 3.2*	27.7 \pm 5.9	5.5–19.5
Neutrophils ($\times 10^6/\mu\text{L}$)	10.8 \pm 2.4†	23.5 \pm 5.1	2.5–12.5

* \dagger Value differs significantly (* $P < 0.001$; † $P = 0.003$) from value after inoculation (paired t test).
 ALT = Alkaline transaminase. ALP = Alkaline phosphatase.
 GGT = γ -Glutamyltransferase.

increased concentrations of BUN and creatinine, marked increases in ALP activity, hyperbilirubinemia, and an increase in anion gap (data not shown).

Serologic testing—At necropsy, 3 dogs inoculated with strain 82 had titers (2 dogs at 1:25 and 1 dog at 1:50). Three dogs inoculated with strain RM 52 had titers of 1:25 on day 14, but agglutination was not evident in samples obtained from 2 of these dogs at subsequent time points. None of the other dogs inoculated with strain RM 52 had agglutinating antibody titers of $> 1:25$.

Discussion

In the study reported here, 2 isolates of *L. kirschneri* serovar grippotyphosa were used to inoculate young, seronegative dogs. One isolate (strain RM 52) was derived from swine tissues, whereas the other isolate (strain 82) was derived from the urine of a dog with clinical leptospirosis. Clinical signs, onset of disease, and gross and microscopic lesions were similar between dogs infected with the 2 isolates of serovar grippotyphosa, but fewer dogs were infected after inoculation with the swine-derived isolate (strain RM 52; 3 of 14 dogs) than after inoculation with the canine-derived isolate (strain 82; 12 of 12 dogs). Hamster passage was used to restore virulence prior to inoculation of dogs, but adaptation of strain RM 52 to culture conditions after multiple passages may have resulted in the loss of factors related to virulence in dogs.²⁰ Alternatively, the swine-derived strain may never have been as virulent for dogs. Cultured leptospires can express different proteins from those for host-derived leptospires.¹⁰ Change in the expression patterns of proteins associated with bacterial entry of strain RM 52 after repeated culture is 1 explanation for the fewer number of dogs infected after inoculation with this organism. Strain 82 organisms were passaged in culture only once since isolation from the original dog in 1992, which may have allowed retention of the characteristics that enabled it to infect the original host.

Signs of leptospirosis in dogs are often nonspecific and may include fever, myalgia, anorexia, vomiting, and diarrhea^{21,22}; furthermore, clinical signs may vary

depending on the infecting serovar. Seven days after inoculation, signs were mild and limited to lethargy and mild scleral hyperemia. Dogs that became infected developed severe clinical signs as early as 9 days after initial inoculation, with clinical illness worsening and resulting in the need for dogs to be euthanized up to day 13. Other signs in acutely ill dogs in the 9- to 13-day postinoculation period included lethargy, vomiting, and anorexia. Fever was detected in only 3 dogs, all of which were inoculated with strain 82. A greater incidence of febrile dogs was expected because fever is commonly listed as a sign of leptospirosis. Because body temperature was measured only twice daily, it is possible that a transient febrile period was missed in some dogs.

In another study,⁵ acute renal failure was a result of infection with serovar grippotyphosa, but hepatic disease appeared to be a minor component. In the study reported here, serum biochemical analyses and histologic lesions of acutely ill dogs were indicative of severe renal and hepatic disease. Seven days after initial inoculation, lesions were evident in the kidneys and liver. There were small multifocal lymphoplasmacytic interstitial infiltrates in the kidneys and perivascular infiltrates of lymphocytes, plasma cells, and fewer neutrophils in the liver. Histologic lesions in acutely ill dogs on days 9 to 13 after inoculation were more severe, with renal lesions consisting of more extensive lymphoplasmacytic interstitial nephritis, tubular dilatation with flattened epithelium (a sign of tubular repair after epithelial cell loss), and multifocal tubular mineralization.

Immunoreactivity to serovar grippotyphosa lipopolysaccharide was detected in small quantities in lumens of the renal tubules and interstitial vessels. Staining was less intense than in dogs in another study.¹⁹ The amount of immunoreactivity may have been related to duration of infection, but the duration of infection in a retrospective study¹⁹ in which investigators applied this antibody to canine tissues was not known. In the liver, hepatocytes had lost glycogen stores; contained numerous small, clear vacuoles (lipid); and were dissociated from hepatic laminae. Multifocal bile plugs were evident, and there were increased numbers of lymphocytes and neutrophils around hepatic portal veins and spread diffusely throughout the sinusoids. One dog had an intussusception, a finding that has been described^{22,24} in animals with leptospirosis.

An increase in values of several biochemical variables in nonrecovering dogs indicated renal and hepatic damage and acid-base abnormalities. There were significant increases in BUN, creatinine, and phosphorus concentrations in samples collected at the time of necropsy, compared with baseline values, indicating that $> 75\%$ of the renal parenchyma was affected. Increases in ALT activity indicate leakage of hepatocytes, and marked increases in ALP and GGT activity and bilirubin concentration indicate cholestasis. Bile flow was most likely disrupted by the dissociation of hepatocytes from hepatic laminae and swelling of hepatocytes as a result of lipid accumulation. An increase in the anion gap and decrease in TCO₂ concentration indicated metabolic acidosis,

probably titrational with loss of bicarbonate attributable to overabundance of uremic acids. Two acutely ill dogs that had been inoculated with strain RM 52 were markedly hyperkalemic as a result of potassium redistribution and failure of renal excretion of potassium (anuria or oliguria). Redistribution during acidosis results when hydrogen ions move intracellularly and potassium ions become extracellular, resulting in increased extracellular potassium concentrations.²⁵

Dogs inoculated with strain RM 52 and euthanatized on days 14, 22, or 28 did not have lesions, except for 2 dogs with mild perivascular hepatic infiltrates. These dogs did not have evidence of infection on the basis of results of leptospiral culture, immunofluorescence testing, or serologic testing. It is possible that organisms never entered the vasculature at the mucous membrane surface, or organisms entered the bloodstream but were cleared before attempts were made to culture leptospire from blood samples. In another study,²⁶ investigators documented that leptospire can be rapidly phagocytosed by tissue macrophages after inoculation. Because control dogs did not have lesions consistent with those of the inoculated dogs, these mild lesions in the kidneys and liver may have been the result of tissue damage attributable to leptospire transiently in those tissues prior to phagocytosis.

Serologic tests for leptospirosis in dogs include the MAT⁹ and ELISA.²⁷ The MAT is commonly used as a diagnostic test in clinical situations, so serum samples of all inoculated dogs were analyzed, when available. Results of serologic tests can be confounded by several factors. Serologic testing is complicated by antibody cross-reactivity among serovars and low titers during acute disease.^{3,5,7} Vaccinated dogs may have an increase in titers that further complicates diagnosis, and some dogs may become infected and actively shed organisms without ever having a titer > 1:100,²⁸ whereas others become seronegative after appropriate treatment.²⁹ We did not detect a serologic titer > 1:50 for any dog inoculated with either strain of serovar grippotyphosa in our study. The most likely explanation for this result is the short time frame between inoculation and the onset of clinical illness because all dogs confirmed infected on the basis of results of leptospiral culture and immunofluorescence testing were euthanatized and necropsied by 13 days after inoculation.

To better understand the course of clinical disease and biochemical abnormalities in dogs infected with leptospirosis, we inoculated 26 dogs with *L. kirschneri* serovar grippotyphosa. To the authors' knowledge, this is the first study to provide results of experimental inoculation with serovar grippotyphosa via the mucous membranes. Two strains of serovar grippotyphosa were used (strain RM 52 was a swine-derived isolate, and strain 82 was a canine-derived isolate). Biochemical changes, gross and histologic lesions, and positive results of leptospiral culture and immunofluorescence testing were evident in a higher number of dogs inoculated with the canine isolate. However, the character of gross, histologic, and biochemical lesions was similar in time course and severity in dogs inoculated with either strain. Evaluation of lesions and clinicopathologic data suggested that infection with serovar grippo-

typhosa results in severe renal and hepatic damage. The reason for the differing rate of infection between these 2 strains of serovar grippotyphosa is not clear but may be attributable to changes in the original host (canine vs swine), altered expression of bacterial proteins resulting from culture passage, or differences in isolates from differing geographic areas. Because of the efficient rate of infection and consistent production of hepatic and renal lesions, strain 82 would make a good candidate for use in additional studies of treatment and preventative measures for acute leptospirosis in dogs.

^aLasix, Patheon Pharmaceuticals Inc, Cincinnati, Ohio.

^bBeuthanasia-D Special, Schering-Plough Animal Health Inc, Kenilworth, NJ.

^c5-fluorouracil, Sigma-Aldrich Corp, St Louis, Mo.

^dPolysorbate leptospira medium 5, Interger Co, Purchase, NY.

^eDehydrated culture media agar (purified); Becton-Dickinson, Franklin Lakes, NJ.

^fBovine serum albumin, Sigma Chemical Co, St Louis, Mo.

^gTriton-X 100, Fisher Chemicals, Fairlawn, NJ.

^hNormal blocking serum (donkey), Vector Laboratories Inc, Burlingame, Calif.

ⁱVector elite ABC kit (1:600) Vector Laboratories Inc, Burlingame, Calif.

^jNOVA RED, Vector Laboratories Inc, Burlingame, Calif.

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