Clinical and Pathologic Comparison of Acute Leptospirosis in Dogs Caused by Two Strains of Leptospirosis Kirschneri Serovar Grippotyphosa

Justin Jay Greenlee  
United States Department of Agriculture

Carole Ann Bolin  
United States Department of Agriculture

David P. Alt  
United States Department of Agriculture

Norman Cheville  
Iowa State University

Claire B. Andreasen  
Iowa State University, candreas@iastate.edu

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Clinical and Pathologic Comparison of Acute Leptospirosis in Dogs Caused by Two Strains of Leptospirosis Kirschneri Serovar Grippotyphosa

Abstract
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 X 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 perivasculitis. 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.

Keywords
acute leptospirosis, conjunctival exposure, serovar, canicola, icterohaemorrhagiae

Disciplines
Small or Companion Animal Medicine | Veterinary Infectious Diseases | Veterinary Pathology and Pathobiology

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

Justin J. Greenlee, DVM, PhD; Carole A. Bolin, DVM, PhD; David P. Alt, DVM, PhD; Norman F. Cheville, DVM, PhD; Claire B. Andreasen, DVM, PhD

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**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.

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**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. The diagnosis of leptospirosis in dogs in the United States and Canada increased substantially between 1983 and 1998. 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, and the use of vaccines containing these serovars has markedly reduced the incidence of disease attributable to them. 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. 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 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. 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), were used in the study. Dogs were housed in pairs in temperature-controlled rooms...
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 refreshed 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.

**Leptospires**—Two strains of *L. hirschneri* 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 leptospires. To prevent loss of virulence through adaptation to culture conditions, leptospires were passaged through hamsters2 and reisolated. Hamsters were housed at the National Animal Disease Center and received IP inoculations consisting of 10^6 organisms. Liver homogenate obtained from a hamster with clinical signs of disease was cultured in 80-40 semi-solid 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, isosthenuria (specific gravity, 1.008), and a titer of 1:1,600 for serovar grippotyphosa. In addition, urine samples obtained from that dog contained leptospires, as determined by use of immunofluorescence testing. This isolate was also obtained from that dog contained leptospires, as determined by immunofluorescence testing. Samples of the liver, kidneys, and urine were collected and placed in neutral-buffered 10% formalin. Samples of the eyes and conjunctiva were collected and placed in Bouin’s solution.

**Experimental design**—All inoculations were administered via conjunctival instillation. Inoculum was prepared as described elsewhere.12 Each inoculum consisted of 5 × 10^7 leptospires in 500 µL of 80–40 semisolid leptospiral culture medium12,13; 250 µL of 80–40 semisolid leptospiral culture medium13; 250 µL of 80–40 lactalbumin hydrolysate was used as described elsewhere,13 with slight modifications (5-fluorouracil 15,c was used at a rate of 100 µg/mL, and nalidixic acid was not used elsewhere,13 with slight modifications (5-fluorouracil15) was used at a rate of 100 mg/mL, and nalidixic acid was not used). Eblinghausen-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.12 A commercially available polysorbate leptospiral medium was used with the addition of purified agar 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 plates of culturing. When cultures became negative, 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.17

Immunofluorescence testing—Samples of the kidneys, liver, urine, aqueous humor, and liver were stained with fluorescein-labeled rabbit anti-leptospiral conjugate, as described elsewhere.10 Leptospires 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 leptosporal 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 with 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, 0.4% Triton X-100, and 1.5% normal blocking serum) 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 complex was added, and sections were incubated for 1 hour at room temperature. Enzyme reactions were accomplished by use of a substrate kit 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 leptosporal culture—Dogs were considered infected when leptospires were detected in tissues or body fluids by use of leptosporal culture, immunofluorescence testing, or immunohistochemical analysis. Results of leptosporal 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°C 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-
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, kidneys, 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.

<table>
<thead>
<tr>
<th>Group</th>
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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. tIncludes data from 1 dog that died and had autolytic tissues that precluded assessment of lesions. tIncludes 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.
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 inoculated 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,
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). 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, 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. The amount of immunoreactivity may have been related to duration of infection, but the duration of infection in a retrospective study 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 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 euthanized 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 leptospires from blood samples. In another study,26 investigators documented that leptospires can be rapidly phagocyted 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 leptospires transiently in those tissues prior to phagocytosis.

Serologic tests for leptospirosis in dogs include the MAT27 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.55 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, 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 euthanized 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 grippotyphosa 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.

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