Detection of asymptomatic renal *Leptospira* infection in abattoir slaughtered cattle in southeastern Georgia, United States

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Abstract

Objectives: Leptospirosis is one of the most widespread zoonotic infectious diseases affecting humans and animals. Several animal species, including cattle, can act as potential asymptomatic carriers facilitating zoonotic transmission of *Leptospira*. This study was conducted to assess the occurrence of asymptomatic renal *Leptospira* carriers among cattle slaughtered in southeastern Georgia, United States.

Methods: A battery of diagnostic tests, including dark field microscopy, direct fluorescent antibody staining, polymerase chain reaction, and culture, were performed on a set of bovine kidneys (n = 37) collected from an abattoir in southeastern Georgia, United States. Virulence of a field isolate obtained from this study was tested in a hamster experimental model.

Results: Motile spirochete-like structures were observed by dark field microscopy in 23 (59%) out of 37 kidney samples tested. In all, 29 samples (78%) were positive by direct fluorescent antibody staining. Only 11 (29.7%) samples by polymerase chain reaction and 3 (8.1%) by culture were positive for *Leptospira* sp. The isolates obtained by culture were confirmed as *Leptospira borgpetersenii*. Hamsters experimentally infected with one of the *Leptospira* field isolates obtained from this study did not show clinical signs but developed renal infection with interstitial nephritis and tubular necrosis.

Conclusions: This study confirms that asymptomatic *Leptospira* renal infection is present among cattle in the region. Our findings underscore the need for future studies to assess the potential environmental contamination and transmission to humans in contact with infected cattle.

Keywords

Leptospira, cattle, diagnosis, public health

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Introduction

Leptospirosis is a reemerging zoonotic disease affecting both humans and animals and is caused by various serovars of *Leptospira*.[1] Asymptomatic renal infection occurs in many domestic and wild animal species. Exposure to urine from infected animals and the contaminated environment results in infection in susceptible humans and animals leading to subclinical mild illness to serious life-threatening disease. Severe and untreated infection may result in medically expensive complications due to renal failure, hepatic disease, pulmonary hemorrhage, and mortality.[1–3] Diagnosis of clinical leptospirosis can be challenging due to its protean manifestations resembling other febrile diseases such as influenza, dengue fever, and rickettsial infections.

The incidence of leptospirosis in humans is increasing globally.[2,3] Although the disease was initially recognized as an occupational disease, it is now considered an important

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reemerging zoonotic disease affecting a broader category of the population. Growth and spread of human populations, deforestation and urbanization, global climate change, ecotourism, and the increase in wildlife and pet trade might favor the increased incidence of the disease.[3–5] In the United States, most of the recent outbreaks were associated with water-related recreational events.[5–7] Abundance of animal reservoirs such as cattle and an ecosystem favoring the maintenance of organisms in the environment will likely increase the exposure and infection rate in susceptible hosts.

Leptospirosis was first reported in cattle in United States in 1946 as outbreaks of severe febrile illness.[8] It is an economically significant disease in terms of cattle production, as the main manifestations include abortions, stillbirth, infertility, and loss of milk production.[9–11] *Leptospira borgpetersenii* (*L. borgpetersenii*) serovar Hardjo is the most common host-adapted *Leptospira* serovar of cattle in North America.[9,10] Many recent studies on prevalence of *Leptospira* infection in cattle are solely based on the seroprevalence.[9–11] Abattoir-based studies on *Leptospira* renal infection date back to the 1990s.[12,13] Serological evaluations do not provide accurate estimates of active infection or carrier status. Therefore, a preliminary study was conducted to evaluate the presence of asymptomatic *Leptospira* renal infection in cattle using kidney samples obtained from a local abattoir using a combination of diagnostic methods.

Materials and methods

Fresh kidneys were collected from cattle slaughtered in a local abattoir. Only 1–2 fresh bovine kidney samples were obtained at a time and processed within 2–4 h of collection in order to improve the detection and isolation rate. Cattle slaughtered in this abattoir came from small independent farms located in southeastern counties in the state of Georgia and from northern Florida. Data on sex, age, and county information were recorded when available. Of the 37 kidney samples, 24 (64.9%) were collected from steers, 5 (13.5%) were collected from bulls, 3 (8.1%) were collected from heifers, and the sex of 5 (13.5%) animals was unknown. In all, 21 (56.8%) cattle were <30 months old, 6 (16.2%) were 30 months or older, and the age of 10 (27.0%) animals was unknown. With respect to the breed, 6 (16.2%) animals were Angus, 1 (2.7%) was Holstein, 1 (2.7%) was Jersey, 1 (2.7%) was a Texas Longhorn, and the breed of 28 (75.7%) animals was unknown. The samples were tested by dark field microscopy (DFM), direct fluorescent antibody staining (DFA), polymerase chain reaction (PCR), and culture. Out of the 37 samples, 3 samples were not examined by DFM and 2 samples were not examined by DFA.

Approximately a 2-inch wedge-shaped sections of kidney encompassing portions of cortex and medulla were processed using aseptic techniques. After removing the capsule, the edges were trimmed and the samples were lightly seared on the surface. Samples were cut into small pieces in a sterile

petri dish using a sterile scalpel blade and were mixed with 10 mL of Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid media (Becton and Dickinson Microbiology Systems, Sparks, MD). The homogenate was allowed to settle for 2–3 m. Supernatant of 15 μL was placed on a glass slide under a coverslip and immediately examined by DFM. Samples containing organisms with morphology and motility compatible with *Leptospira* were recorded as positive. A total of 15 μL of the sample was placed on circles of a glass slide and processed for DFA as described previously.[14] Briefly, smears were allowed to air-dry, fixed in chilled acetone for 15 m and treated with anti-*Leptospira* polyclonal antibody conjugated with fluorescein isothiocyanate (National Veterinary Services Laboratory, Ames, IA) for 1.5 h at 37 °C. The slides were washed three times in phosphate buffered saline (PBS) and air-dried. The slides were examined under 40× objective of a fluorescent microscope after applying the mounting fluid and a coverslip. The samples with typical morphology of *Leptospira* and positive fluorescence were recorded as positive.

Culture was performed as described previously with modifications.[15] The kidney samples were homogenized as described above and 500 μL of the supernatant from the kidney homogenate was added to 9.5 mL P80-BA liquid oleic media.(National Veterinary Services Laboratory, Ames, IA).. Three serial 10-fold dilutions (10^{-2}, 10^{-3}, and 10^{-4}) were prepared, and 100 μL from the 10^{-2} dilution was inoculated into two semisolid P-80-BA liquid oleic media tubes with 5-fluorouracil and two without 5-fluorouracil. The tubes were incubated at 29 °C up to 6 months. The samples were examined for the presence of *Leptospira* at 2-week intervals by DFM. An aliquot of the homogenate was stored in a −70 °C freezer for PCR. DNA was extracted in an automated DNA extraction unit (BioSprint-96, Qiagen Inc, Valencia, CA) using MagMAX™ total nucleic acid isolation kit (Applied Biosystems, Foster City, CA) using manufacturer's recommendations. Real-time PCR was conducted using a previously described protocol.[16]

Leptospira isolates obtained by culture were subcultured into semisolid and liquid P-80-BA liquid oleic media. DNA was isolated using QIAamp DNA Mini Kit (QIAGEN Inc, Valencia, CA). In order to further characterize the isolates, PCR was performed targeting Lip L32 (present in all pathogenic *Leptospira* spp.), IS*1500* (specific for *L. interrogans*), and IS*1533*(specific for *L. borgpetersenii*) gene sequences.[17–19]

To evaluate the virulence of the *Leptospira* isolates, a preliminary experimental inoculation study was conducted in hamsters using one of the field isolates as described previously.[20] Animals were kept following institutional animal care and protocols approved by the University of Georgia's Institutional Animal Care and Use Committee (IACUC). Four hamsters were inoculated intraperitoneally with 1 mL of *Leptospira* isolate (BK6) containing 1×10^7 organisms. One control hamster was inoculated with P-80-BA liquid oleic

Table 1. Summary of *Leptospira* testing results from 37 bovine kidney samples obtained from a local abattoir in southeastern Georgia, United States.

Test	Positive	Negative	Not evaluated	Total
DFM	23 (62.2%)	11 (29.7%)	3 (8.1%)	37 (100%)
DFA	30 (81.1%)	5 (13.5%)	2 (5.4%)	37 (100%)
PCR	11 (29.7%)	26 (70.3%)	0 (0.0%)	37 (100%)
Culture	3 (8.1%)	34 (91.9%)	0 (0.0%)	37 (100%)

DFM: dark field microscopy; DFA: direct fluorescent antibody test; PCR: polymerase chain reaction.

media. The hamsters were observed twice daily for the development of clinical signs. After 28 days postinoculation, the hamsters were humanely euthanized, necropsy was conducted, and the organs were collected in 10% neutral buffered formalin and processed for routine histopathology using standard protocols. The kidney samples were cultured on P-80-BA liquid oleic media. Microscopic agglutination testing (MAT) on the hamster serum was performed against six *Leptospira* serovars.

Results

Motile spirochete-like structures were observed by DFM in 23 out of 34 (59%) kidney samples examined. In all, 29 (78%) samples out of 35 tested were positive by DFA. Of 37 kidney samples tested, 11 (29.7%) samples were positive by PCR and 3 (8.1%) samples were positive by culture. A summary of the results for each test is shown in Table 1. Two *Leptospira* isolates were obtained from steers under 30 months of age, and the age and sex of the animal that contributed to the third isolate were unavailable. The origin of one isolate is unknown. The cycle threshold (Ct) values for 11 real-time PCR positive samples ranged from 31.63 to 39.64 with an average Ct value of 35.20. The *Leptospira* isolates obtained were also positive for the screening PCR used in the study. PCR for the Lip L32 gene, which is present in all pathogenic *Leptospira*, was positive for all three isolates (Figure 1(a)). PCR for insertion sequences IS*1500* and IS*1533* was performed to differentiate *L. borgpetersenii* and *L. interrogans*. All three isolates were positive for IS*533* (Figure 1(b)) and negative for IS*1500* (Figure 1(c)) confirming the isolates as *L. borgpetersenii*.

Experimental inoculation of hamsters with one of the isolates did not induce any clinical signs. The hamsters remained asymptomatic during the entire duration of the study. No gross lesions were observed at necropsy 28 days postinoculation. Histopathological examination revealed multifocal renal tubular ectasia, necrosis, and mild interstitial nephritis in all inoculated hamsters (Figures 2(a) and (b)). The control hamster did not develop any renal lesions. The inoculated hamsters developed an antibody response (MAT = 1/400) only to the serovar Hardjo among the six routinely tested
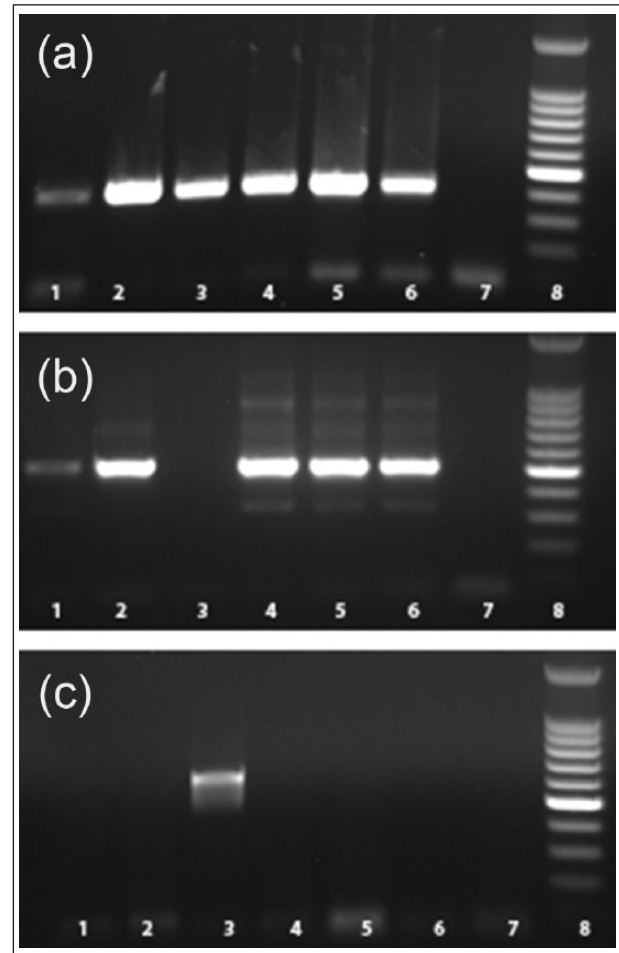

Figure 1. Polymerase chain reaction using field isolates: (a) Lip L32 gene, (b) IS*1533* gene, (c) IS*1500* gene. Lane 1: *L. borgpetersenii* serovar Hardjo type A (NVSL S 1343); Lane 2: *L. borgpetersenii* serovar Hardjo type B (NVSL S 818); Lane 3: *L. interrogans* serovar Hardjo; Lane 4: field isolate BK6; Lane 5: field isolate BK9; Lane 6: field isolate BK30; Lane 7: field isolate BK6; Lane 8: negative control; Lane 9: molecular weight markers. NVSL: National Veterinary Services Laboratory.

serovars in our laboratory. The *Leptospira* organisms were recovered from kidneys of all infected hamsters by culture.

Discussion

This study emphasizes that *Leptospira borgpetersenii* serovar Hardjo infection is prevalent in cattle in the study area. A combination of diagnostic tests was applied to the kidney samples in this study to improve the sensitivity of detection. We observed more samples positive by DFA and DFM than culture and PCR combined. It is important to note that the DFM and DFA are not specific for the detection of the pathogenic *Leptospira*, and the possibility of false-positive results cannot be excluded.

Three *L. borgpetersenii* serovar Hardjo isolates were obtained from this study. *Leptospira* culture is not generally
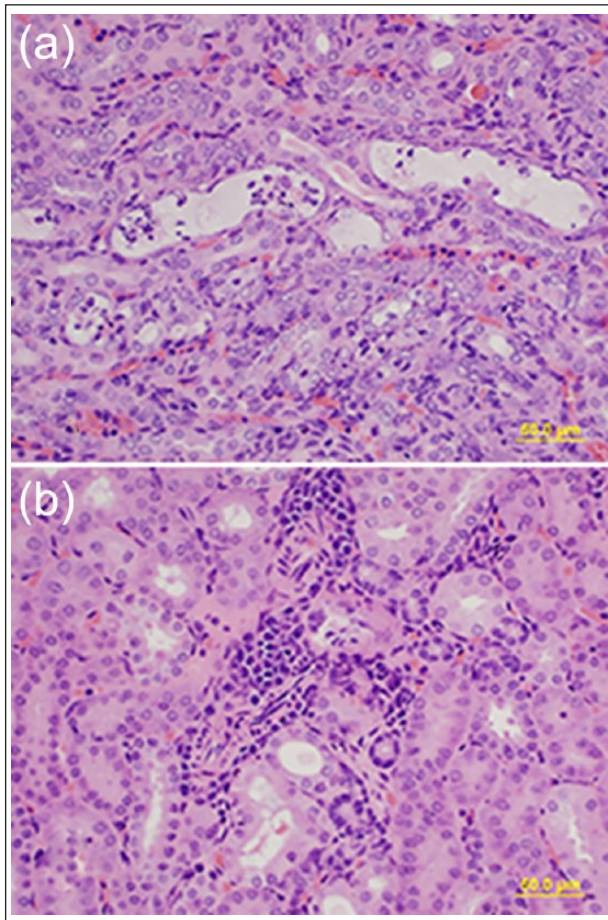
Figure 2. Representative hematoxylin and eosin stained sections of hamster kidney infected with field isolate BK6, 400× magnifications. Note (a) the tubular ectasia and necrosis and (b) interstitial nephritis, bar = 100 μm

attempted in diagnostic laboratories due to its laborious nature, long periods of incubation, and contamination with other fast-growing bacteria. *L. borgpetersenii* serovar Hardjo is a very slow-growing and hard to maintain species, and special media (PA-80-BA liquid oleic media) were needed to grow and maintain cultures. The diagnostic complexity due to the presence of large number of serovars and animal reservoirs emphasizes the need of culture to obtain *Leptospira* isolates for future epidemiologic evaluations and strategic implementation of preventive measures. *L. borgpetersenii* serovar Hardjo types A and B are reported in the North American cattle population.[21,22]*Leptospira* isolates belonging to serovars Pomona and Grippotyphosa have also been isolated from cattle.[12,13] In a nationwide abattoir-based study conducted in 1990, the majority of the isolates were *L. borgpetersenii* serovar Hardjo type A based on restriction endonuclease analysis.[12,13] Most of the type B *L. borgpetersenii* serovar Hardjo isolates from cattle were isolated from the southeastern United States, while type A did not have any particular pattern of distribution. In a recent study which compared virulence of *L.*

borgpetersenii serovar Hardjo A and B types in hamsters, the type A isolate established chronic renal colonization, whereas the type B isolate induced severe debilitating illness.[23] The field isolate tested in our study resulted in renal infection in hamsters without inducing clinical signs as described with the type A isolates. Characterization of these *L. borgpetersenii* serovar Hardjo isolates is ongoing. Genomic sequencing data have suggested that this *L. borgpetersenii* serovar Hardjo has undergone a considerable genome reduction resulting in reduced indirect transmission potential.[24] However, it is important to note that *L. borgpetersenii* serovar Hardjo was the most commonly diagnosed serovar from notified cases of human leptospirosis in New Zealand.[25,26]

Leptospirosis is an important occupational disease in New Zealand among abattoir workers.[27] Elimination or reduction of *L. borgpetersenii* serovar Hardjo infection in cattle populations is a desirable goal due to its economic and public health impact, but currently available vaccines do not provide sterile immunity or prevent establishment of renal carriers.[23] Many aspects of cattle *L. borgpetersenii* serovar Hardjo infection, including the establishment of infection, variation in disease patterns, immune response generated, the mode of transmission and maintenance of infection, and the impact of various strains in relation to ecology and management systems remain largely unknown. In a previous study, we conducted in dairy cattle in this region, 7 out of 10 dairy herds in the region tested positive for *Leptospira* in urine by DFA, and all the herds tested had at least one cow with antibody titers (by MAT) ≥100 for one or more *Leptospira* serovars.[14] Life-threatening disease and mortality due to leptospirosis in dogs are reported in this region.[28] Moreover, kidneys of multiple wild animal species (bobcats, coyotes, and opossums) tested were PCR positive for *Leptospira* indicating widespread distribution of animal reservoirs (S. Rajeev, unpublished data).

Our study indicates an endemic distribution of *L. borgpetersenii* serovar Hardjo in cattle populations in the region. Most of Georgia, especially the southeastern region has a subtropical, humid climate with moderate to heavy precipitation rate promoting an environmental maintenance of *Leptospira*. Additionally, there are numerous water bodies that are frequently used for recreation and irrigation. In humans, leptospirosis is a treatable illness if diagnosed early; however, accurate diagnosis is challenging due to its confounding clinical presentations, lack of awareness and clinical suspicion among patients and physicians, and limited diagnostic expertise at the point of care. As the majority of *Leptospira* cases are mild and may get misdiagnosed as febrile illness such as influenza, it is likely that its incidence in humans is underreported. Considering the abundance of animal reservoirs as shown in our data, changing climatic conditions of the region and the overall global trends in human leptospirosis incidence, studies to explore the distribution and extent of human exposure to *Leptospira* spp. in the southeastern United States are warranted.

References

1. Levett PN. Leptospirosis. *Clin Microbiol Rev* 2001; 14: 296–326.
2. McBride AJ, Athanazio DA, Reis MG, et al. Leptospirosis. *Curr Opin Infect Dis* 2005; 18: 376–386.
3. Guerra MA. Leptospirosis: public health perspectives. *Biologicals* 2013; 41: 295–297.
4. Vinetz JM, Glass GE, Flexner CE, et al. Sporadic urban leptospirosis. *Ann Intern Med* 1996; 125: 794–798.
5. Morgan J, Bornstein SL, Karpati AM, et al. Outbreak of leptospirosis among triathlon participants and community residents in Springfield, Illinois, 1998. *Clin Infect Dis* 2002; 34: 1593–1599.
6. Stern EJ, Galloway R, Shadomy SV, et al. Outbreak of leptospirosis among Adventure Race participants in Florida, 2005. *Clin Infect Dis* 2010; 50: 843–849.
7. Meites E, Jay MT, Deresinski S, et al. Reemerging leptospirosis, California. *Emerg Infect Dis* 2004; 10: 406–412.
8. Mathews FP. A contagious disease of cattle associated with Leptospira. *Am J Vet Res* 1946; 7: 78–93.
9. Grooms DL and Bolin CA. Diagnosis of fetal loss caused by bovine viral diarrhea virus and Leptospira spp. *Vet Clin North Am Food Anim Pract* 2005; 21: 463–472.
10. Grooms DL. Reproductive losses caused by bovine viral diarrhea virus and leptospirosis. *Theriogenology* 2006; 66: 624–628.
11. Guitian J, Thurmond MC and Hietala SK. Infertility and abortion among first-lactation dairy cows seropositive or seronegative for Leptospira interrogans serovar hardjo. *J Am Vet Med Assoc* 1999; 215: 515–518.
12. Miller DA, Wilson MA and Beran GW. Relationships between prevalence of Leptospira interrogans in cattle, and regional, climatic, and seasonal factors. *Am J Vet Res* 1991; 52: 1766–1768.
13. Miller DA, Wilson MA and Beran GW. Survey to estimate prevalence of Leptospira interrogans infection in mature cattle in the United States. *Am J Vet Res* 1991; 52: 1761–1765.
14. Rajeev S, Berghaus RD, Overton MW, et al. Comparison of fluorescent antibody and microscopic agglutination testing for Leptospira in pregnant and nonpregnant cows. *J Vet Diagn Invest* 2010; 22: 51–54.
15. Zuerner RL. Laboratory maintenance of pathogenic Leptospira. *Curr Protoc Microbiol* 2005; Chapter 12: Unit 12E.1.
16. Smythe LD, Smith IL, Smith GA, et al. A quantitative PCR (TaqMan) assay for pathogenic Leptospira spp. *BMC Infect Dis* 2002; 2: 13.
17. Levett PN, Morey RE, Galloway RL, et al. Detection of pathogenic leptospires by real-time quantitative PCR. *J Med Microbiol* 2005; 54: 45–49.
18. Zuerner RL, Alt D and Bolin CA. IS1533-based PCR assay for identification of Leptospira interrogans sensu lato serovars. *J Clin Microbiol* 1995; 33: 3284–3289.
19. Zuerner RL and Bolin CA. Differentiation of Leptospira interrogans isolates by IS1500 hybridization and PCR assays. *J Clin Microbiol* 1997; 35: 2612–2617.
20. Haake DA. Hamster model of leptospirosis. *Curr Protoc Microbiol* 2006; Chapter 12: Unit 12E.2.
21. Ellis WA, Thiermann AB, Montgomery J, et al. Restriction endonuclease analysis of Leptospira interrogans serovar hardjo isolates from cattle. *Res Vet Sci* 1988; 44: 375–379.
22. Marshall RB, Winter PJ, Thiermann AB, et al. Genotypes of Leptospira interrogans serovar hardjo in cattle in the UK. *Vet Rec* 1985; 117: 669–670.
23. Zuerner RL, Alt DP and Palmer MV. Development of chronic and acute golden Syrian hamster infection models with Leptospira borgpetersenii serovar Hardjo. *Vet Pathol* 2012; 49: 403–411.
24. Bulach DM, Zuerner RL, Wilson P, et al. Genome reduction in Leptospira borgpetersenii reflects limited transmission potential. *Proc Natl Acad Sci U S A* 2006; 103: 14560–14565.
25. Benschop J, Heuer C, Jaros P, et al. Sero-prevalence of leptospirosis in workers at a New Zealand slaughterhouse. *N Z Med J* 2009; 122: 39–47.
26. Dreyfus A, Benschop J, Collins-Emerson J, et al. Seroprevalence and risk factors for leptospirosis in abattoir workers in New Zealand. *Int J Environ Res Public Health* 2014; 11: 1756–1775.
27. Mansell C and Benschop J. Leptospirosis is an important multi-species zoonotic disease in New Zealand. *N Z Med J* 2014; 127: 5–8.
28. Rajeev S, Woldemeskel MW and Westmoreland DS. Pathology in practice. Leptospirosis. *J Am Vet Med Assoc* 2012; 240: 957–959.