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Brief Original Article

Detection and genotyping of *Leptospira* spp. from the kidneys of a seemingly healthy pig slaughtered for human consumption

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Abstract

Introduction: Leptospirosis is a zoonotic disease caused by pathogenic *Leptospira* spp. Leptospirosis is maintained in an environment due to chronic kidney infection of a wide variety of domestic, peridomestic and wild reservoir mammals. In this study the role of pigs in maintenance of leptospires on the Caribbean island of St. Kitts was investigated.

Methodology: The condemned kidneys of 60 pigs slaughtered at a St. Kitts abattoir were screened by a quantitative-PCR for the presence of *Leptospira* spp. Positive samples were genotyped using a six-gene based multilocus sequence typing scheme.

Results: Leptospiral DNA was detected in the kidneys of one of the 60 pigs. Multilocus sequence typing identified the infecting species to be *L. interrogans*.

Conclusions: Detection of this zoonotic pathogen in the kidneys of a seemingly healthy pig raises concerns regarding the subclinical carriers of the disease among the island's swine population.

Key words: Leptospirosis; *Leptospira*; swine; genotyping; Multi-locus sequence typing (MLST).

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Introduction

Leptospirosis is a highly prevalent zoonotic disease caused by pathogenic *Leptospira* species. Leptospirosis affects over 150 species of animals, including humans, dogs, cattle, horses and pigs. The pathogen persists in the kidneys of reservoir animals and is shed in the urine. Susceptible hosts acquire the infection following exposure to infected animal urine or *Leptospira*-contaminated water or soil [1,2]. The Caribbean region is considered to be an endemic area for leptospirosis with a high incidence rate [3].. Recently, we showed that one-fifth of tested water sources on the island of St. Kitts were contaminated with leptospiral DNA [4]. Pigs are important source of meat on the island with approximately 2000 pigs slaughtered annually for local consumption. Pigs are primarily raised by landless livestock farmers who own a small number of animals and use traditional management practices, which include outdoor rearing, grazing and slaughtering of the older pigs. To understand the role pigs might play in maintenance of leptospirosis on the island, we collected condemned kidneys from 60 pigs slaughtered over a period of one year at a St. Kitts abattoir and screened for the presence of leptospiral DNA.

Methodology

Kidneys were condemned on the basis of the macroscopic criteria proposed by Baker *et al.* [5] namely the presence of foci of pale or red discoloration scattered throughout the cortical surface. A part of each kidney was fixed in 10% neutral-buffered formalin, routinely processed and embedded in paraffin wax, cut at 5µm and stained with hematoxylin and eosin (HE) for histological evaluation. DNA was isolated from unfixed, frozen or fresh kidney tissue (20mg) using a DNeasy Blood and Tissue Mini-Kit (Qiagen, Valencia, USA), following the manufacturer's directions.

A TaqMan qPCR targeting leptospiral *lipl32* gene was used to screen DNA extracted from sixty pigs (kidney samples =114), as described previously [6]. The assay was performed on an ABI 7500 (Applied Biosystems, Foster City, USA) using Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, USA) and thermal conditions of a holding stage of 95^{o}C for 20 seconds, and 40 cycles of 95^{o}C

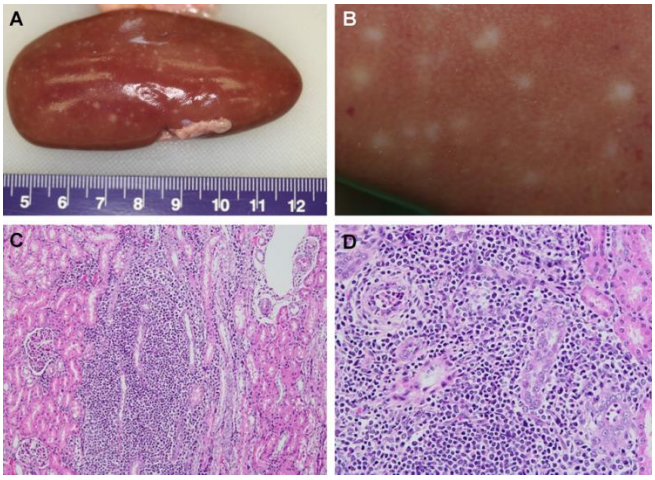
for 3 seconds and 60°C for 30 seconds. Each plate contained DNA equivalent to 10^5, 10^4, 10^3, 10^2, 10, 1, 0.1, and 0.01 leptospiral genome units. Genomic standards were prepared from cultured *L. interrogans*, and genome equivalents were calculated as described previously [7]. Each column, except positive control columns, had a no-template control. Reactions were performed in 25 µL final volumes, using 5 µL of extracted DNA, 500nM of LipL32-45F (forward primer; 5'- AAGCATTACCGCTTGTGGTG-3'), 500 nM of LipL32-286R (reverse primer; 5'-GAACTCCCATTTCAGCGATT-3') and 100 nM of LipL32-189P (probe; FAM-5'-AAAGCCAGGACAAGCGCCG-3'-BHQ1) [6]. For analysis, a threshold of 100,000 and baseline between 5 and 12 cycles were used. Samples with consistent results between duplicates and Ct < 40 were considered positive. Samples that were undetectable or had a Ct of > 40 were considered negative. A run was considered valid only if all ten no-template controls were negative [4].

To genotype the infecting *Leptospira* spp., a six-gene multilocus sequence typing (MLST) scheme based on *adk*, *icdA*, *secY*, *rrs2*, *lipL41*, and *lipL32* genes was used [8]. These loci were PCR amplified from the *Leptospira* in the positive sample, designated SKIT_AV_Po, using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA), and previously described primers and thermal conditions [8,9]. PCR products from *icdA*, *secY*, *rrs2*, *lipL41*, and *lipL32* genes were gel extracted using QIAquick gel extraction kit (Qiagen, Valencia, USA) and sequenced in a commercial facility (Davis Sequencing, Davis, USA). Nucleotide sequences deposited in the GenBank database have accession numbers KM211315, KM211316, KM211317, KM211318, KM211319. Phylogenetic analyses were performed by the Neighbor-Joining method (10) using MEGA (v6.06) software. The trees were statistically supported by bootstrapping with 1000 replicates, and phylogenetic distances were measured by the Kimura two-parameter model.

Results and Discussion

Out of 114 swine kidney samples screened, two were positive by this assay. The two positive samples, RL7C and RL7M had a Ct value of 23.12 and 26.5, and contained $1.5x10^5$ and $1.8x10^4$ genome units/20mg of kidney tissue, respectively. Both these samples came from the cortical and medullary regions of the same kidney. Grossly, the affected kidney was characterized by poorly defined, multifocal areas of pale-tan discoloration, ranging from 2 to 5 mm in size, scattered throughout the cortex ('white-spotted' appearance) [2]. Microscopically, sections of the kidney revealed multiple foci of perivascular and interstitial lymphoplasmacytic inflammatory cell infiltration within the cortex and a relatively prominent focus of lymphoplasmacytic tubulo-interstitial nephritis near the cortico-medullary junction (Figure 1).

Figure 1. A and **B**. Multifocal areas of discoloration scattered throughout the renal cortex. **C** and **D**. Multiple foci of perivascular and interstitial lymphoplasmacytic inflammatory cell infiltration within the cortex and a relatively prominent focus of lymphoplasmacytic tubulo-interstitial nephritis near the cortico-medullary junction, 20× and 40×, HE.



For genotyping of the positive sample, we were able to PCR amplify all but the *adk* gene. Primers for the *adk* gene non-specifically amplified a porcine gene, which suggests that the described primers and/or thermal conditions for PCR amplification of *adk* gene from porcine samples need further standardization. All five genes showed 100% identity with *L. interrogans* in BLAST searches using the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/). By phylogenetic analysis, the *icdA*, *lipl32*, *lipl41*, *rrs2 and secY* genes of leptospiral strain SKIT_AV_Po clustered very closely with cognate genes of *L. interrogans* strains [Supplemental Fig. S1 (A)-(F)]. In all the five genes, *L. interrogans* strains detected in humans in the Andaman and Nicobar Islands, India, appeared to be the nearest neighbor of strain SKIT_AV_Po.

This is the first report of identification of a circulating species of *Leptospira* on St. Kitts. Although subclinical infections are common in pigs, the detection of this zoonotic pathogen in the kidneys

of an animal destined for human consumption raises concerns about its public health implications. One of the short-comings of this study was that we did not use a host housekeeping gene as a control for PCR inhibitors, which may have resulted in some false negative results.

Conclusions

Detection of this zoonotic pathogen in the kidneys of a seemingly healthy pig raises concerns regarding the subclinical carriers of the disease among the island's swine population. Larger multi-species studies are required to estimate the true prevalence of the disease, and the roles of wild and domestic animals in the transmission cycle of leptospirosis on the island.

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References

1 Ko AI, Goarant C, Picardeau M (2009) *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat Rev Microbiol 7: 736-747.
2. Adler B, de la Peña Moctezuma A (2010) *Leptospira* and leptospirosis. Vet Microbiol 140: 287-296.
3. Pappas G, Papdimitriou P, Siozopoulou V, Christou L, Akritidis N (2008) The globalization of leptospirosis: worldwide incidence trends. Int J Infect Dis 12: 351-357.
4. Rawlins J, Portanova A, Zuckerman I, Loftis A, Ceccato P, Willingham AL, Verma A. Molecular detection of leptospiral DNA in environmental water on St. Kitts. Int J Environ Res Public Health, in press.
5. Baker TF, McEwen SA, Prescott JF, Meek AH (1989) The prevalence of leptospirosis and its association with multifocal interstitial nephritis in swine at slaughter. Can J Vet Res 53: 290-294.
6. Stoddard RA, Gee JE, Wilkins PP, McCaustland K, Hoffmaster AR (2009) Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the LipL32 gene. Diagn Microbiol Infect Dis 64: 247-255.
7 Levett PN, Morey RE, Galloway RL, Turner DE, Steigerwalt AG, Mayer LW (2005) Detection of pathogenic leptospires by real-time quantitative PCR. J Med Microbiol 54: 45-49.
8 Ahmed N, Devi SM, Valverde Mde L, Vijayachari P, Machang'u RS, Ellis WA, Hartskeerl RA (2006) Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* species. Ann Clin Microbiol Antimicrob 5: 28.
9 Ahmed A, Thaipadungpanit J, Boonsilp S, Wuthiekanun V, Nalam K, Spratt BG, Aanensen DM, Smythe LD, Ahmed N, Feil EJ, Hartskeerl RA, Peacock SJ (2011) Comparison of two multilocus sequence based genotyping schemes for *Leptospira* species. PLoS Negl Trop Dis. 5: e1374.
10 Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

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Conflict of interests: No conflict of interests is declared.

Supplementary Items

Supplemental figure S1 (A)-(F). Phylogenetic analysis of the partial nucleotide sequences of the *icdA, lipl32, lipl41, rrs2* and *secY* genes of *Leptospira* strain SKIT_AV_Po with cognate genes of other *Leptospira* strains. In all trees, the position of strain SKIT_AV_Po is highlighted by a closed blue square.