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BRIEF REPORT

Detection and quantification of leptospires in urine of dogs: a maintenance host for the zoonotic disease leptospirosis

P. Rojas · A. M. Monahan · S. Schuller · I. S. Miller · B. K. Markey · J. E. Nally

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Abstract Leptospirosis is a global zoonotic disease. Pathogenic *Leptospira* species, the causative agent of leptospirosis, colonize the renal tubules of chronically infected maintenance hosts such as dogs, rats and cattle. Maintenance hosts typically remain clinically asymptomatic and shed leptospires into the environment via urine. In contrast, accidental hosts such as humans can suffer severe acute forms of the disease. Infection results from direct contact with infected urine or indirectly, through contaminated water sources. In this study, a quantitative real-time PCR specific for *lipL32* was designed to detect the urinary shedding of leptospires from dogs. The sensitivity and specificity of the assay was evaluated using both a panel of pathogenic *Leptospira* species and clinical microbial isolates, and samples of urine collected from experimentally infected rats and non-infected controls. The lower limit of detection was approximately 3 genome equivalents per reaction. The assay was applied to canine urine samples collected from local dog sanctuaries and the University Veterinary Hospital (UVH) at University College Dublin. Of 525 canine urine samples assayed, 37 were positive, indicating a prevalence of urinary shedding of leptospires of 7.05%. These results highlight the need to provide effective canine vaccination strategies and raise public health awareness.

P. Rojas · A. M. Monahan · S. Schuller · I. S. Miller · B. K. Markey · J. E. Nally
School of Agriculture, Food Science & Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland

J. E. Nally (✉)
Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland
e-mail: Jarlath.Nally@ucd.ie

Leptospires colonize the renal tubules of maintenance host species such as dogs, rats and cattle. They are excreted via urine into the environment, where they can survive in suitable moist conditions. Maintenance hosts are typically asymptomatic whilst accidental hosts can suffer a wide range of clinical manifestations including hepatic and renal failure, and severe pulmonary haemorrhage [1]. Contact with infected urine or contaminated water sources may result in infection since leptospires can enter the body through broken skin or mucosal surfaces such as the conjunctival tissue of the eye [2].

Specific serovars of *Leptospira* are associated with asymptomatic carriage and persistent shedding in urine, in particular mammalian host species [3–5]. Leptospiruria in maintenance hosts is of high intensity, constant and of long duration compared to accidental hosts where it is of low intensity, intermittent and of short duration [6]. Such host adaptation was first described by Babudieri who observed ‘biological equilibrium’ between rodent hosts and certain *Leptospira* serovars [7].

Dogs can act as a maintenance host for pathogenic *Leptospira* serovars. Historically, canine leptospirosis has been associated with serovars Canicola and Icterohaemorrhagiae, but canine serum may contain antibodies specific for a wide range of serovars including Autumnalis, Bratislava, Grippotyphosa, Hardjo, Pomona, and Zanoni [8–13]. Clinical presentation of acute disease depends in part on the infecting serovar and can range from mild to severe [14]. Clinical resolution of acute infection may lead to asymptomatic shedding. In addition, there is evidence to suggest that clinically normal dogs can be chronic carriers of infection, and thus maintenance hosts, shedding leptospires via urine into the environment [15–17]. Serological surveys often report more than 20% of examined canine sera contain antibodies specific for pathogenic *Leptospira* serovars. However, it is difficult to correlate serological

findings with the prevalence of chronic infection and leptospiruria, which has been reported in the presence of relatively low or negative antibody titres [3, 18].

In this study, a real-time PCR assay was designed to target a 57 bp amplicon within *lipL32*, a gene that encodes an outer membrane lipoprotein present in pathogenic leptospires but absent in saprophytic species [19, 20]. The *lipL32* gene sequence is highly conserved amongst pathogenic leptospires and displays 96.9% average pairwise DNA sequence identity [21]. The nucleotide sequences for *lipL32* from 36 submissions (accession numbers listed below) to the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), comprising six species and more than 17 serovars of leptospires, were aligned using Mega 4 [22]. Primer Express software (Perkin Elmer Applied Biosystems) was used to design primers which were subsequently modified to include degenerate bases, and accommodate all sequence alignments (forward primer, 5'- TCGCTGAAATRGGWGTTCGT-3'; reverse primer, 5'- CGCCTGGYTCMCCGATT-3'). A fluorescent probe (FAM 5'- ATTTCCCCAACAGGCG-3' NFQ) was designed which was specific for a consensus region of the resulting 57 bp amplicon. Specificity of primers and probe was confirmed by BLAST (http://blast.ncbi.nlm.nih.gov/). Quantitative real-time PCR was performed using an Applied Bioscience 7500 Thermo cycler, with a single starting cycle of 50°C for two minutes, then a 95°C step for ten minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Each reaction comprised 25 μL final volume: 12.5 μL of Taqman mastermix (Applied Biosystems), 1 μL of labelled probe (250 μM), 3 μL of each primer (600 nM) (Sigma Aldrich), 1 μL of ultra pure water and 4.5 μL of template. Each unknown sample was tested in triplicate and each dilution of the standard curve in duplicate. Each run included a non-template control, a positive control urine sample from experimentally infected rats and a negative control.

The use of Taqman chemistry provided additional sensitivity and specificity for PCR amplicons by inclusion of a 16 bp *lipL32* gene specific fluorescent probe which was conserved amongst all aligned sequences, and representative of six different species of pathogenic leptospires. The incorporation of two degenerate bases into forward and reverse primers permitted consensus amongst all aligned sequences, and successful amplification from all serovars tested. For example, *lipL32* gene sequence derived from *L. interrogans* serovar Icterohaemorrhagiae (AY423075.1), *Leptospira interrogans* serovar Canicola (AY763509.1), *Leptospira interrogans* serovar Tarassovi (AY609330.1) and *L. interrogans* serovar Pomona (AY223718.1) differ at the base pair positions replaced with degenerate bases. DNA from all pathogenic serovars of *Leptospira* tested positive by the *lipL32* real-time PCR assay (Table 1). In contrast, DNA derived from the saprophytic serovar Patoc and seventeen additional clinical microbial isolates obtained from the Bacteriology Diagnostic Laboratory at the University College Dublin Veterinary Hospital were negative.

In order to emulate as closely as possible a typical clinical urine sample, and the effects of typical inhibitors of PCR associated with urine, a standard curve to determine the efficiency of the *lipL32* real-time PCR was generated by spiking non-infected rat urine with known amounts of a virulent strain of *L. interrogans* serovar Copenhageni prior to DNA extraction. A standard curve was generated as follows: 10 mL of *L. interrogans* serovar Copenhageni (4.3×10^8 leptospires/mL) was centrifuged for ten minutes at 10,000 × *g* at 4°C. The resulting pellet was resuspended in 1 mL of urine from non-infected rats and ten-fold serial dilutions performed in negative urine. Each dilution was centrifuged at 10,000 × *g* at 4°C, followed by DNA extraction. DNA was extracted using a Qiagen Mini Kit with a Qiagen Vacuum Manifold, as per manufacturer's instructions with slight modification. Samples were digested with proteinase K for 2 hours. A positive (urine collected from an experimentally infected rat) and negative control were included for extraction in each processed batch of 22 samples. The number of leptospires (assuming one genome equivalent per leptospire) was calculated based on the extracted DNA concentration obtained with a fluorospectrometer (Nanodrop ND 1000, Coleman Technologies, V3.5.2). The efficiency of the standard curve was 98.5% (Fig. 1). The lowest detectable number of genome equivalents was 2.59 per reaction, equivalent to 172.6 leptospires/mL of urine, assuming one

Table 1 List of serovars of *Leptospira* tested by the *lipL32* real-time PCR assay

Serogroup	Serovar	Strain
Autumnalis	Autumnalis	Akyami
Ballum	Ballum	Mus 127
Bataviae	Bataviae	Kariadi-Satu
Bataviae	Bataviae	Swart
Semaranga[a]	Patoc[a]	Patoc 1[a]
Australis	Bratislava	B2a
Canicola	Canicola	Hond Utrecht IV
Icterohaemorrhagiae	Copenhageni	RJ16441
Icterohaemorrhagiae	Icterohaemorrhagiae	Lai
Icterohaemorrhagiae	Lai Type Langkawi	Langkawi
Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
Pomona	Pomona	Pomona
Sejroe	Hardjo Type Bovis	Hardjobovis
Tarassovi	Tarassovi	Mitis Johnson

[a] All strains are pathogenic except for the saprophytic serovar Patoc.

genome equivalent/leptospire (Fig. 2); thus demonstrating the sensitivity of the assay with clinically relevant criteria.

Urine samples collected from experimentally infected rats were used to further demonstrate the sensitivity and specificity of the *lipL32* real-time PCR assay. Experimentally infected rats remain clinically asymptomatic but shed large numbers of leptospires in urine approximately 10–14 days post-infection [23–25]. Wistar male *Rattus norvegicus* (University College Dublin, Biomedical Facility), 150–210 g, 6 weeks of age, were experimentally infected as previously described [23]. Rats were housed in metabolism cages once weekly and 200 µL urine collected for enumeration of leptospires by quantitative real-time PCR. All study protocols were approved by the University College Dublin Animal Research Ethics Committee and conducted under license from the Department of Health and Children. All urine samples collected from experimentally infected rats were positive by the *lipL32* real-time PCR assay by two weeks post-infection, and remained positive up to six weeks post-infection. In contrast, urine samples collected at day 0, week 1 and from all negative control rats remained negative. The sensitivity and specificity of the *lipL32* real-time PCR assay is therefore validated by the lack of any positive amplification in non-infected control rats, and perhaps more importantly, by the lack of any positive amplification in urine samples from experimentally infected rats at day 0 or at week 1 post-infection. This suggests that these rats were infected but leptospires were not yet being excreted in urine from colonized renal tubules.

The *lipL32* real-time PCR was used to assess canine urine samples submitted to a diagnostic laboratory (UVH) for routine urinalysis. It was also used for samples collected by free catch from local dog sanctuaries. A 2-mL aliquot of urine was collected from 498 canine urine samples, routinely submitted for urinalysis to the University Veterinary hospital (UVH) in University College Dublin (UCD). A further 27 canine urine samples were collected from dogs in sanctuaries by free catch, with sample volumes ranging from 0.35 to 35 mL. All collected samples were stored at 4°C for no more than 24 hours, centrifuged at 10,000 × *g* for ten minutes and the resulting pellets stored at −20°C prior to analysis. Canine urine samples were considered positive when at least 2/3 of the Ct values were lower than that of the Ct value of the lowest dilution of the standard curve. In addition, all canine urine pellets from dog sanctuaries were examined by dark-field microscopy. Of 498 samples collected from the UVH and tested for the presence of *lipL32*, 34 (6.83%) were positive (Table 2). Of the 34 positive samples, the average number of genome equivalents of leptospires shed per ml of urine was 6.22×10^4, and ranged from as few as 35.5 leptospires per ml

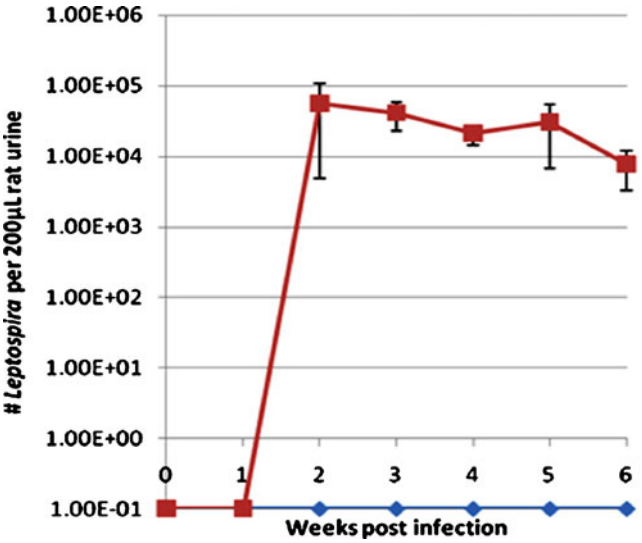

Fig. 2 Detection and quantification of *Leptospira* by the *lipL32* real-time PCR assay in urine samples collected from experimentally infected rats (n=8, *squares*) and non-infected controls (n=4, *diamonds*)

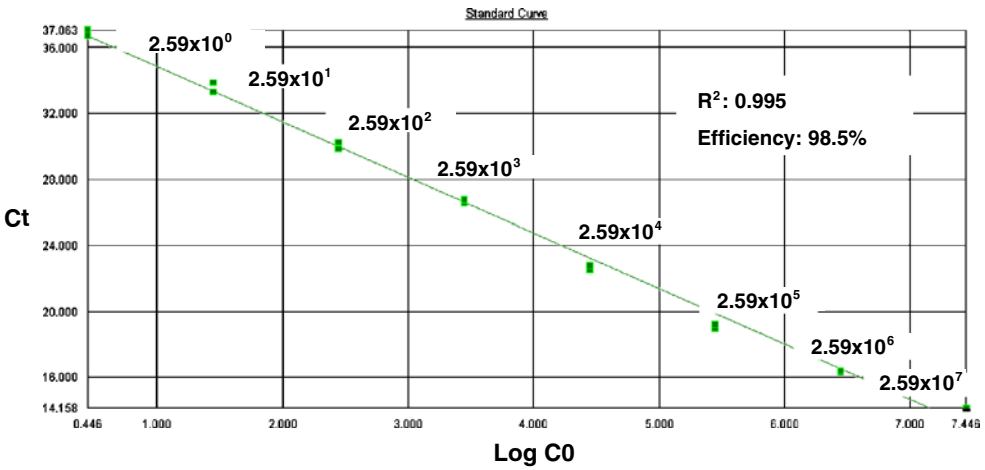

Fig. 1 Standard curve of the *lipL32* real-time PCR assay using DNA extracted from ten-fold serial dilutions of in vitro cultivated *Leptospira* diluted in negative rat urine

Table 2 Detection of pathogenic leptospires in canine urine samples in Ireland

Sample source	Number of samples	Number of positive	% positive	Number of leptospires/mL urine
UVH	498	34	6.83	Range = $35.5–1.33\times10^6$; mean = 6.22×10^4
Sanctuaries	27	3[a]	11.11	Range = $76.1–4.46\times10^4$; mean = 2.3×10^4
Total	525	37	7.05	Range = $35.5–1.33\times10^6$; mean = 5.9×10^4

Numbers of samples tested are provided, as are numbers of samples that were positive by the *lipL32* real-time PCR

[a] A single canine urine sample from a dog sanctuary was also positive by dark-field microscopy for the presence of spirochaetes

to 1.33×10^6 leptospires per mL of urine collected. Out of 27 samples collected from local dog sanctuaries, 3 (11.11%) were positive. The average number of genome equivalents of leptospires shed per mL of urine was 2.3×10^4, and ranged from as few as 76.1 leptospires per mL to 4.46×10^4 leptospires per mL of urine collected. In addition, one of the three samples was positive for the presence of spirochaetes by dark field microscopy (Table 2). None of the canine urine samples from sanctuaries cultured positive.

Whilst a larger number of samples collected from dog sanctuaries were positive (11.1%) compared to samples from UVH (6.83%), insufficient numbers of samples were collected from dog sanctuaries to determine if this was a significant difference. Further, larger volumes of urine samples were collected by free catch from sanctuaries (up to 35 ml) compared to UVH samples (limited to 2 ml). Clinical examination of dogs in local dog sanctuaries indicated that all dogs were clinically normal (data not shown). Vaccination status was not available in order to determine whether any of the positive urine samples came from dogs vaccinated against leptospirosis.

An internal amplification control was not included in this assay. Rather, for every batch of clinical urine samples from which DNA was extracted, a positive urine sample from an experimentally infected rat was extracted, as well as a negative control. Each *lipL32* real-time assay plate contained each of these controls, as well as each dilution of the standard curve, which were performed in duplicate, whilst unknowns were performed in triplicate.

Results suggest that 7.05% of domestic dogs in Ireland shed leptospires in urine. Leptospiruria has also been demonstrated in 8.8% of dogs in the United States and 22% of dogs in Iran by routine PCR and nested PCR of 23 S and 16 S gene sequence, respectively [15, 17]. Results highlight the global significance of canine leptospirosis and its zoonotic potential. The lower prevalence of leptospiruria in our study may reflect the smaller volume tested (2 ml compared to 6–20 ml) or the specificity for *lipL32* compared to 23 S and 16 S encoding genes. Quantification of numbers of leptospires in each positive sample indicated that several dogs excreted more than 10^4 leptospires/mL urine. Despite repeated attempts, culture of leptospires from canine urine was unsuccessful and thus typing of excreted leptospires was not possible. It will be important in future studies to determine the species and serovar identity of leptospires excreted in such samples to assess zoonotic risk and to further understand canine leptospirosis.

Accession numbers of *lipL32* gene sequences used for alignments

AF366366, AM937000.1, AY461905.1, AY763509.1, AY609322.1, AY423075.1, AY609330.1, AY461901.1, EU871716.1, AY609326.1, AY609327.1, DQ149595.1, AY776294.1, AY609333.1, AY609329.1, U89708.1, AF181553.1, AF121192.1, AF181556.1, AF181555.1, AY461910.1, AY776293.1, AY223718.1, AY461904.1, EU871720.1, EU871719.1, EU871718.1, AY461908.1, AY461907.1, AY461906.1, EU871723.1, AY461899.1, AY461903.1, AY609328.1, AY609331.1, AE016823.1.

Panel of clinical microbial isolates tested for specificity

Bacillus cereus, Bordetella bronchiseptica, Candida albicans, Citrobacter freundii, Enterobacter aerogenes, Enterococcus faecalis, Escherichia coli, Hyphomicrobium species, *Klebsiella pneumoniae, Listeria monocytogenes, Pasteurella multicocida, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella enterica serovar* Dublin, *Staphylococcus aureus, Staphylococcus epidermidis, Yersinia enterocolitica.*

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