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

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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 validated 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.

Dear Dr. Van Belkum,

Thank you for considering our manuscript entitled “Detection and quantification of leptospire in urine of dogs; a maintenance host for the zoonotic disease leptospirosis” for publication in the European Journal of Clinical Microbiology and Infectious Diseases. I would also like to thank the reviewer for constructive critique. Please find below a response to each point raised by the reviewer; a revised manuscript has been prepared accordingly and is provided for your consideration for publication as a brief report.

Reviewer #2:

Comment: This manuscript has improved with the changes made, however I was expecting to see a concise communication, but the manuscript still appears as a full research paper.

Response: The manuscript has been revised, as also requested by the editors, as a brief report.

Comment: Some justification for the choice of specificity test organisms is provided in the cover letter; however, I still have concerns with regard to those tested. Are these organisms those that would normally be encountered as potential pathogens in dogs, especially from urine samples?

Response: The reviewer is correct; these are microbes routinely encountered either as pathogens or contaminants in submitted canine urine samples and are routinely found in diagnostic laboratories.

Comment: The Patoc 1 strain only appears in table 1, but should also appear in the list given on page 8.

Response: This now appears on the list indicated by the reviewer.

Comment: Further to the Sybr Green assay reported by Levett et al, there is also another base upon TaqMan chemistry by Stoddard et al. (Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the LipL32 gene. *Diagn Microbiol Infect Dis* 2009 Jul;64(3):247-55 (ISSN: 1879-0070) Stoddard RA; Gee JE; Wilkins PP; McCaustland K; Hoffmaster AR). This should be mentioned in the discussion.

Response: This reference has been added to the manuscript.

Minor Points:

Comment: The results, page 9, line 6: *in vitro* should appear in italics.

Response: Modified as suggested.

Comment: Table 1 - the symbol used in the table to denote Patoc 1 does not appear in the key.

Response: Modified as suggested.

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

Running title: Detection of pathogenic *Leptospira* in canine urine

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1 **Abstract**

2 Leptospirosis is a global zoonotic disease. Pathogenic *Leptospira* species, the causative
3 agent of leptospirosis, colonize the renal tubules of chronically infected maintenance hosts
4 such as dogs, rats and cattle. Maintenance hosts typically remain clinically asymptomatic
5 and shed leptospire into the environment via urine. In contrast, accidental hosts such as
6 humans can suffer severe acute forms of the disease. Infection results from direct contact
7 with infected urine or indirectly, through contaminated water sources. In this study, a
8 quantitative Real-Time PCR specific for *lipL32* was designed to detect the urinary shedding
9 of leptospire from dogs. The sensitivity and specificity of the assay was evaluated using
10 both a panel of pathogenic *Leptospira* species and clinical microbial isolates, and samples
11 of urine collected from experimentally infected rats and non-infected controls. The lower
12 limit of detection was approximately 3 genome equivalents per reaction. The assay was
13 applied to canine urine samples collected from local dog sanctuaries and the University
14 Veterinary Hospital (UVH) at University College Dublin. Of 525 canine urine samples
15 assayed, 37 were positive, indicating a prevalence of urinary shedding of leptospire of
16 7.05%. These results highlight the need to provide effective canine vaccination strategies
17 and raise public health awareness.

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1 **Text**

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

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

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

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

4 In this study, a Real-Time PCR assay was designed to target a 57bp amplicon within
5 *lipL32*, a gene that encodes an outer membrane lipoprotein present in pathogenic
6 leptospires but absent in saprophytic species [19, 20]. The *lipL32* gene sequence is highly
7 conserved amongst pathogenic leptospires, and displays 96.9% average pairwise DNA
8 sequence identity [21]. The nucleotide sequences for *lipL32* from 36 submissions
9 (accession numbers listed below) to the National Center for Biotechnology Information
10 (www.ncbi.nlm.nih.gov), comprising 6 species and more than 17 serovars of leptospires,
11 were aligned using Mega 4 [22]. Primer Express software (Perkin Elmer Applied
12 Biosystems) was used to design primers which were subsequently modified to include
13 degenerate bases, and accommodate all sequence alignments (forward primer; 5'-
14 TCGCTGAAATRGGWGTTTCGT-3', reverse primer 5'- CGCCTGGYTCMCCGATT-3').
15 A fluorescent probe (FAM 5'- ATTTCCCCAACAGGCG-3' NFQ) was designed which
16 was specific for a consensus region of the resulting 57bp amplicon. Specificity of primers
17 and probe was confirmed by BLAST (<http://blast.ncbi.nlm.nih.gov/>). Quantitative Real-
18 Time PCR was performed using an Applied Bioscience 7500 Thermo cycler, with a single
19 starting cycle of 50°C for 2 minutes, then a 95°C step for 10 minutes followed by 40 cycles
20 of 95°C for 15 seconds and 60°C for 60 seconds. Each reaction comprised 25µL final
21 volume; 12.5µL of Taqman mastermix (Applied Biosystems), 1µL of labelled probe
22 (250µM), 3µL of each primer (600nM) (Sigma Aldrich), 1µL of ultra pure water and 4.5µL
23 of template. Each unknown sample was tested in triplicate and each dilution of the standard

1 curve in duplicate. Each run included a non-template control, a positive control urine
2 sample from experimentally infected rats and a negative control.

3 The use of Taqman chemistry provided additional sensitivity and specificity for
4 PCR amplicons by inclusion of a 16bp *lipL32* gene specific fluorescent probe which was
5 conserved amongst all aligned sequences, and representative of 6 different species of
6 pathogenic leptospires. The incorporation of two degenerate bases into forward and reverse
7 primers permitted consensus amongst all aligned sequences, and successful amplification
8 from all serovars tested. For example, *lipL32* gene sequence derived from *L. interrogans*
9 serovar Icterohaemorrhagiae (AY423075.1), *Leptospira interrogans* serovar Canicola
10 (AY763509.1), *Leptospira interrogans* serovar Tarassovi (AY609330.1) and *L. interrogans*
11 serovar Pomona (AY223718.1) differ at the base pair positions replaced with degenerate
12 bases. DNA from all pathogenic serovars of *Leptospira* tested positive by the *lipL32* Real-
13 Time PCR assay (Table 1). In contrast, DNA derived the saprophytic serovar Patoc and
14 seventeen additional clinical microbial isolates obtained from the Bacteriology Diagnostic
15 Laboratory at the University College Dublin Veterinary Hospital were negative.

16 In order to emulate as closely as possible a typical clinical urine sample, and the
17 effects of typical inhibitors of PCR associated with urine, a standard curve to determine the
18 efficiency of the *lipL32* Real-Time PCR was generated by spiking non-infected rat urine
19 with known amounts of a virulent strain of *L. interrogans* serovar Copenhageni prior to
20 DNA extraction. A standard curve was generated as follows: 10mL of *L. interrogans*
21 serovar Copenhageni (4.3×10^8 leptospires/mL) was centrifuged for 10 minutes at 10,000 x
22 g at 4°C. The resulting pellet was resuspended in 1mL of urine from non-infected rats and
23 10 fold serial dilutions performed in negative urine. Each dilution was centrifuged at
24 10,000 x g at 4°C, followed by DNA extraction. DNA was extracted using a Qiagen Mini

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1 Kit with a Qiagen Vacuum Manifold, as per manufacturer's instructions with slight
2 modification. Samples were digested with proteinase K for 2 hours. A positive (urine
3 collected from an experimentally infected rat) and negative control were included for
4 extraction in each processed batch of 22 samples. The number of leptospire (assuming one
5 genome equivalent per leptospire) was calculated based on the extracted DNA
6 concentration obtained with a fluorospectrometer (Nanodrop ND 1000, Coleman
7 Technologies, V3.5.2). The efficiency of the standard curve was 98.5% (Figure 1). The
8 lowest detectable number of genomes equivalents was 2.59 per reaction, equivalent to
9 172.6 leptospire/mL of urine, assuming one genome equivalent/leptospire (Figure 1), thus
10 demonstrating the sensitivity of the assay with clinically relevant criteria.

11 Urine samples collected from experimentally infected rats were used to further
12 demonstrate the sensitivity and specificity of the *lipL32* Real-Time PCR assay.
13 Experimentally infected rats remain clinically asymptomatic but shed large numbers of
14 leptospire in urine approximately 10-14 days post-infection [23-25]. Wistar male *Rattus*
15 *norvegicus* (University College Dublin, Biomedical Facility), 150-210 g, 6 weeks of age,
16 were experimentally infected as previously described [23]. Rats were housed in metabolism
17 cages once weekly and 200µL urine collected for enumeration of leptospire by
18 quantitative Real-Time PCR. All study protocols were approved by the University College
19 Dublin Animal Research Ethics Committee and conducted under license from the
20 Department of Health and Children. All urine samples collected from experimentally
21 infected rats were positive by the *lipL32* Real-Time PCR assay by 2 weeks post-infection,
22 and remained positive up to 6 weeks post-infection. In contrast, urine samples collected at
23 day 0, week 1 and from all negative control rats remained negative. The sensitivity and
24 specificity of the *lipL32* Real-Time PCR assay is therefore validated by the lack of any

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1 positive amplification in non-infected control rats, and perhaps more importantly, by the
2 lack of any positive amplification in urine samples from experimentally infected rats at day
3 0 or at week 1 post-infection. This suggests that these rats were infected but leptospire
4 were not yet being excreted in urine from colonized renal tubules.

5 The *lipL32* Real-Time PCR was used to assess canine urine samples submitted to a
6 diagnostic laboratory (UVH) for routine urinalysis. It was also used for samples collected
7 by free catch from local dog sanctuaries. A 2mL aliquot of urine was collected from 498
8 canine urine samples, routinely submitted for urinalysis to the University Veterinary
9 hospital (UVH) in University College Dublin (UCD). A further 27 canine urine samples
10 were collected from dogs in sanctuaries by free catch, with sample volumes ranging from
11 0.35 to 35mL. All collected samples were stored at 4°C for no more than 24 hours,
12 centrifuged at 10,000 x g for 10 minutes and the resulting pellets stored at -20°C prior to
13 analysis. Canine urine samples were considered positive when at least 2/3 of the Ct values
14 were lower than that of the Ct value of the lowest dilution of the standard curve. In
15 addition, all canine urine pellets from dog sanctuaries were examined by dark-field
16 microscopy. Of 498 samples collected from the UVH and tested for the presence of *lipL32*,
17 34 (6.83%) were positive (Table 2). Of the 34 positive samples, the average number of
18 genome equivalents of leptospire shed per ml of urine was 6.22×10^4 , and ranged from as
19 few as 35.5 leptospire per ml to 1.33×10^6 leptospire per mL of urine collected. Out of 27
20 samples collected from local dog sanctuaries, 3 (11.11%) were positive. The average
21 number of genome equivalents of leptospire shed per mL of urine was 2.3×10^4 , and ranged
22 from as few as 76.1 leptospire per mL to 4.46×10^4 leptospire per mL of urine collected. In
23 addition, one of the three samples was positive for the presence of spirochaetes by dark

1 field microscopy (Table 2). None of the canine urine samples from sanctuaries cultured
2 positive.

3 Whilst a larger number of samples collected from dog sanctuaries were positive
4 (11.1%) compared to samples from UVH (6.82%), insufficient numbers of samples were
5 collected from dog sanctuaries to determine if this was a significant difference. Further,
6 larger volumes of urine samples were collected by free catch from sanctuaries (up to 35ml)
7 compared to UVH samples (limited to 2ml). Clinical examination of dogs in local dog
8 sanctuaries indicated that all dogs were clinically normal (data not shown). Vaccination
9 status was not available in order to determine whether any of the positive urine samples
10 came from dogs vaccinated against leptospirosis.

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

17 Results suggest that 7.05% of domestic dogs in Ireland shed leptospires in urine.
18 Leptospirosis has also been demonstrated in 8.8% of dogs in the U.S. and 22% of dogs in
19 Iran by routine PCR and nested PCR of 23S and 16S gene sequence respectively [15, 17].
20 Results highlight the global significance of canine leptospirosis and its zoonotic potential.
21 The lower prevalence of Leptospirosis in our study may reflect the smaller volume tested
22 (2ml compared to 6-20ml) or the specificity for *lipL32* compared to 23S and 16S encoding
23 genes. Quantification of numbers of leptospires in each positive sample indicated that
24 several dogs excreted more than 10^4 leptospires/mL urine. Despite repeated attempts,

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1 culture of leptospire from canine urine was unsuccessful and thus typing of excreted
2 leptospire was not possible. It will be important in future studies to determine the species
3 and serovar identity of leptospire excreted in such samples, to assess zoonotic risk, and to
4 further understand canine leptospirosis.

6 **Accession Numbers of *lipL32* gene sequences used for alignments**

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

14 **Panel of clinical microbial isolates tested for specificity**

15 *Bacillus cereus*, *Bordetella bronchiseptica*, *Candida albicans*, *Citrobacter freundii*,
16 *Enterobacter aerogenes*, *Enterococcus faecalis*, *Escherichia coli*, *Hyphomicrobium*
17 *species*, *Klebsiella pneumoniae*, *Leptospira biflexa serovar Patoc*, *Listeria monocytogenes*,
18 *Pasteurella multocida*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella enterica*
19 *serovar Dublin*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Yersinia*
20 *enterocolitica*.

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11 Browne for technical advice, Olwen Golden, and the dog sanctuaries for permission to
12 collect urine samples.

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Serogroup	Serovar	Strain
Autumnalis	Autumnalis	Akyami
Ballum	Ballum	Mus 127
Bataviae	Bataviae	Kariadi-Satu
Bataviae	Bataviae	Swart
*Semaranga	*Patoc	*Patoc 1
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

Table 1 List of serovars of *Leptospira* tested by the *lipL32* Real-Time PCR assay.

*All strains are pathogenic except for the saprophytic serovar Patoc.

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	Number of samples	Number of positive	% positive	Number of leptospire/mL urine
UVH	498	34	6.83	range= 35.5 – 1.33x10 ⁶ ; mean= 6.22x10 ⁴
Sanctuaries	27	3*	11.11	range= 76.1– 4.46x10 ⁴ ; mean= 2.3x10 ⁴
Total	525	37	7.05	range= 35.5 – 1.33x10 ⁶ ; mean= 5.9x10 ⁴

Table 2 Detection of pathogenic leptospire in canine urine samples in Ireland. Numbers of samples tested are provided, as are numbers of samples that were positive by the *lipL32* Real-Time PCR. *A single canine urine sample from a dog sanctuary was also positive by dark-field microscopy for the presence of spirochaetes.

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1 **Figure Legends**

2

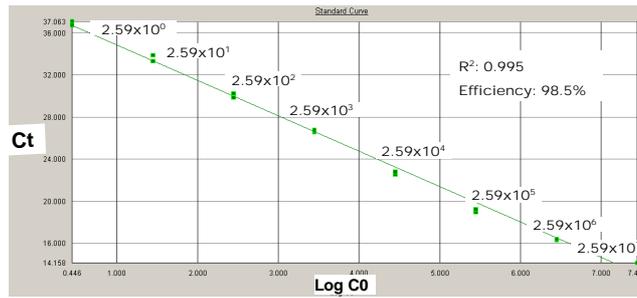
3 **Figure 1** Standard curve of the *lipL32* Real-Time PCR assay using DNA extracted
4 from 10 fold serial dilutions of in *vitro* cultivated *Leptospira* diluted in negative rat urine.

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6 **Figure 2** Detection and quantification of *Leptospira* by the *lipL32* Real-Time PCR
7 assay in urine samples collected from experimentally infected rats (n=8, square) and non-
8 infected controls (n=4, diamond).

9

Rojas et al., Figure 1



Rojas et al., Figure 2

