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TaqMan real-time PCR for detection of pathogenic *Leptospira* spp. in canine clinical samples

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Abstract

Introduction: Canine leptospirosis has always been a differential diagnosis in dogs presenting with clinical signs and blood profiles associated with kidney and/or liver disease. The conventional polymerase chain reaction (PCR) provides diagnoses, but real-time PCR-based tests provide earlier confirmation and determine the severity of infection, especially in the acute stage, allowing early detection for immediate treatment decisions. To our knowledge, real-time PCR has not been routinely adopted for clinical investigation in Malaysia. This study evaluated TaqMan real-time PCR (qPCR) assays diagnosing leptospirosis and compared their applicability to clinical samples from dogs with kidney and/or liver disease against a conventional PCR reference. **Material and Methods:** The qPCR assays were validated using existing leptospiral isolates. Whole blood and urine samples were analysed using a conventional PCR, *LipL32(1)* and *LipL32(2)* qPCRs and a microscopic agglutination test. The sensitivity and specificity of the qPCRs were determined. **Results:** The *LipL32(1)* qPCR assay had more diagnostic value than the *LipL32(2)* qPCR assay. Further evaluation of this assay revealed that it could detect as low as five DNA copies per reaction with high specificity for the tested leptospiral strains. No cross-amplification was observed with other organisms. Analysing the clinical samples, the *LipL32(1)* qPCR assay had 100.0% sensitivity and >75.0% specificity. **Conclusion:** The *LipL32(1)* qPCR assay is sensitive, specific and has the potential to be applied in future studies.

Keywords: canine leptospirosis, qPCR, sensitivity, specificity.

Introduction

Cases of canine leptospirosis have been reported worldwide and were often associated with the Canicola and Icterohaemorrhagiae serovars of *Leptospira* spp., but dogs may be infected with a wide range of serovars or genotypes (3, 37). The clinical presentation of canine leptospirosis ranged from mild to severe signs involving kidney and liver insufficiency and/or pulmonary haemorrhage (9, 17). In clinical cases, an early and accurate diagnosis of suspected leptospirosis in dogs is essential to allow an immediate decision on treatment and achieve a favourable outcome. Therefore, a validated diagnostic tool should be available to confirm a clinical suspicion of leptospirosis, especially when the clinical signs are not specific to the disease (19).

To date, isolation of the bacterium and serology using a microscopic agglutination test (MAT) remain the methods of reaching a definitive diagnosis. However, the isolation of *Leptospira* spp. requires the tedious step of obtaining a pure bacterial culture, which takes months in the laboratory, and isolation is therefore not suitable for detecting leptospirosis in the short acute phase (1). Serologically, MAT requires the maintenance of a panel of live cultured leptospiral antigens in the laboratory, and unlike diagnosis by bacteria isolation, this method benefits canine patients in the acute and convalescent phases (10). As alternatives to these methods, polymerase

chain reaction (PCR) assays as both conventional and real-time (qPCR) variants have been widely used for diagnosing leptospirosis because they have reliable sensitivity. Both PCR assays were used to detect the pathogenic *Leptospira* spp. *LipL32* gene (15, 18, 43). Low cost is an advantage of conventional PCR assays in detecting the *LipL32* gene, therefore these assays are useful for routine clinical diagnosis. On the other hand, detecting the *LipL32* gene using a qPCR has several advantages, including a shorter turnaround time, increased specificity when 5′ nuclease assay probes are used, and substantially less likelihood of cross-contamination (25).

In humans, the qPCR assay has been demonstrated suitable for clinical diagnosis of leptospirosis (23, 36, 40). A similar assay could be adopted for dogs, but the qPCR assay standardised for human clinical samples cannot be used directly for dogs, it needs to be validated using canine clinical samples (35). There is limited information on the analytical sensitivity of the qPCR assay with canine whole blood and urine samples (11, 43). Only one study evaluated the qPCR assay using experimentally contaminated whole blood and urine samples from healthy dogs (25).

In Malaysia, conventional PCRs have been used routinely (16, 21, 32), but the utility of a qPCR assay in detecting pathogenic *Leptospira* spp. in dog samples has not been established locally. Therefore, this experiment evaluated TaqMan qPCR assays and investigated their applicability in the diagnosis of canine leptospirosis compared with the conventional PCR as a reference method in whole blood and urine samples from dogs diagnosed with kidney and/or liver disease.

Material and Methods

Bacterial strains. Thirty-eight leptospiral strains from pathogenic, intermediate and saprophytic groups of seven species (*L. biflexa*, *L. borgpetersenii*, *L. kirschneri*, *L. kmetyi*, *L. weilii*, *L. selangorensis* and *L. wolffii*) that were frequently reported locally were selected and used for primer and TaqMan probe validation and optimisation. The leptospiral strains were provided by the Bacteriology Laboratory, Faculty of Veterinary Medicine (FVM), Universiti Putra Malaysia (UPM) and had been isolated from local environmental and animal samplings. The serovars were maintained in Ellinghausen McCullough Johnson Harris (EMJH) media incubated at 30°C. The species and serovar were identified using partial 16S rRNA sequencing and serotyping, respectively (Table 1). An additional six bacterial isolates (other than *Leptospira*) from clinical specimens provided by the Bacteriology Laboratory (FVM, UPM) were included for primer and TaqMan probe validation (Table 1). The selected bacteria were grown overnight in nutrient broth at 37°C maintained in an aerobic condition. The microbial species were identified using a standard biochemical identification method.

Table 1. List of bacteria used in the qPCR assay

Bacterium (n = 44)	Number tested (n)	Source
Pathogenic group		
L. borgpetersenii serovar Ballum strain Mus 127	1	FVM, UPM
L. borgpetersenii serovar Hardjobovis strain 117123	1	FVM, UPM
L. borgpetersenii serovar Javanica	1	Dog
L. borgpetersenii serovar Javanica strain Veldrat Bataviae 46	1	FVM, UPM
L. borgpetersenii serovar Tarassovi strain Perepelitsin	1	FVM, UPM
L. interrogans serovar Australis	2	Dog
L. interrogans serovar Australis strain Ballico	1	FVM, UPM
L. interrogans serovar Autumnalis strain Akiyami A	1	FVM, UPM
L. interrogans serovar Bataviae	8	Dog
L. interrogans serovar Bataviae strain Swart	1	FVM, UPM
L. interrogans serovar Canicola strain Hond Utrecht IV	1	FVM, UPM
L. interrogans serovar Copenhageni strain M20	1	FVM, UPM
L. interrogans serovar Djasiman strain Djasiman	1	FVM, UPM
L. interrogans serovar Hebdomadis strain Hebdomadis	1	FVM, UPM
L. interrogans serovar Icterohaemorrhagiae strain RGA	1	FVM, UPM
L. interrogans serovar Lai strain Lai	1	FVM, UPM
L. interrogans serovar Pomona strain Pomona	1	FVM, UPM
L. interrogans serovar Pyrogenes strain Salinem	1	FVM, UPM
L. kirschneri serovar Cynopteri strain 3522C	1	FVM, UPM
L. kirschneri serovar Grippotyphosa strain Moskva V	1	FVM, UPM
L. kmetyi serovar Malaysia strain Bejo-ISO9	1	FVM, UPM
L. weilii serovar Celledoni strain Celledoni	1	FVM, UPM
Intermediate group		
L. selangorensis	3	Environmental
L. wolffii	5	Environmental
Saprophytic group		
L. biflexa serovar Patoc strain Patoc I	1	FVM, UPM
Other bacteria		
Bacillus spp. (Gram positive)	1	Clinical
Escherichia coli (Gram negative)	1	Clinical
Pseudomonas aeruginosa (Gram negative)	1	Clinical
Salmonella spp. (Gram negative)	1	Clinical
Staphylococcus aureus (Gram positive)	1	Clinical
Streptococcus spp. (Gram positive)	1	Clinical

FVM – Faculty of Veterinary Medicine; UPM – Universiti Putra Malaysia

Table 2. Primers and TaqMan probes for qPCR of pathogenic *Leptospira* spp.

Target gene	Nucleotide sequence	Reference
LipL32(1)		
Forward	5′-AAG CAT TAC CGC TTG TGG TG-3′	36
Reverse	5′-GAA CTC CCA TTT CAG CGA TT-3′	
TaqMan probe	FAM/ZEN-5'-AA AGC CAG GAC AAG CGC CG-3′-IBFQ	
LipL32(2)		
Forward	5′-CGG GAG GCA GCA GTT AAG AAT-3′	41
Reverse	5′-ACG TAT GGT GCA AGC GTT GTT-3′	
TaqMan probe	FAM/ZEN-5′-GCA ATG TGA TGA TGG TAC CTG CCT-3′-IBFQ	

Sample collection. A total of 124 dogs identified with kidney and/or liver disease presented to a primary veterinary healthcare hospital (University Veterinary Hospital (UVH), FVM, UPM) or private veterinary clinics within a 10 km radius of UPM were recruited. Both diseases simultaneously afflicted 68 dogs, kidney disease was the complaint of 34 dogs, and liver disease affected the remaining 22. There were 102 dogs with acute clinical illness (≤7 days from infection) and 22 dogs with chronic clinical illness (>7 days from infection). The inclusion criteria of the recruited dogs were abnormal and elevated serum biochemistry parameters: urea >7.5 mmol/L, creatinine >176 µmol/L, alanine aminotransferase >90.0 U/L, and alkaline phosphatase >100 U/L. The duration of clinical illness for each dog was recorded and categorised based on published guidelines (13). Whole blood samples (of which 124 were used), serum (of which also 124 were tested) and urine samples (of which 113 were available) were collected from the dogs by experienced veterinarians. Ethical approval for this study was obtained from the Institutional Animal Care and Use Committee (UPM/IACUC/AUP-R084/2016).

Serological detection using a microscopic agglutination test. Microscopic agglutination testing was performed using a panel of 20 leptospiral serovars, namely Australis, Autumnalis, Ballum, Bataviae, Canicola, Celledoni, Copenhageni, Cynopteri, Djasiman, Grippotyphosa, Hardjobovis, Hebdomadis, Icterohaemorrhagiae, Javanica, Lai, Malaysia, Patoc, Pomona, Pyrogenes and Tarassovi. Endpoint titres were determined using serial two-fold dilutions, and the last well with 50.0% agglutination was recorded. The cut-off for a positive MAT reaction was a titre ≥1:100, as defined in previous studies (26, 28), and the serovar with the highest MAT titre was recorded.

Extraction of genomic DNA. Genomic DNA from pure bacterial culture, whole blood and urine was extracted using a DNeasy Blood and Tissue kit (Qiagen, Germantown, MD, USA), and the protocols provided in the kit were used as described. One protocol was for non-nucleated cells and applied to whole blood, and the second protocol was for cultured cells and intended for urine and bacterial cultures. The total genomic DNA of the bacterial culture was quantified using an Infinite M200 Pro multimode plate reader (Tecan, Männedorf, Switzerland). The end products (DNA template) of bacterial culture, whole blood and urine extraction were inspected for purity using 1.5% agarose gels.

Molecular detection using a conventional polymerase chain reaction (PCR). Sets of primers that targeted the 16S rRNA and *LipL32* genes (2, 33) were used. The primer sequences for 16S rRNA were 5'-CAT GCAAGTCAAGCGGAGTA-3' (forward) and 5'-AGT TGAGCCCGCAGTTTTC-3' (reverse). The primer sequences for *LipL32* were 5'-GTCGACATGAAAAAACTTTCGATT TTG-3' (forward) and 5'-CTGCAGTTACTTAGTCGCGTCAGA AGC-3' (reverse). The *LipL32* gene was present only in pathogenic *Leptospira* spp. (38). The reaction volume was 25 µL and was optimised as follows: 12.5 µL 2× MyTaq Red Mix (Bioline, London, UK), 1.25 µL of forward primer and 1.25 µL of reverse primer, and 10 µL of DNA template. Amplification was optimised and performed in a Mastercycler Pro S thermal cycler (Eppendorf, Germany) with initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 45 s, and DNA extension at 72°C for 30 s before the final extension step at 72°C for 6 min. Amplicons were analysed in tris-borate-ethylenediaminetetraacetic acid buffer at 80 V for 1.5 h with 1.5% gel electrophoresis. The gel was pre-stained with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) and examined using an Alphaimager gel documentation system (ProteinSimple, San José, CA, USA). Amplicons were identified by their band sizes at 541 base pairs (bp) (16S rRNA) and 756 bp (*LipL32*).

Primer and TaqMan probe specificity. Two sets of LipL32 primers (*LipL32*(1) and *LipL32*(2)) and TaqMan probes were used in this study (Table 2). All bacterial strains were tested using both sets of primers and respective TaqMan probes to determine the specificity in detecting pathogenic *Leptospira* spp. Pathogenic leptospiral strains were grouped as the positive control, intermediate and saprophytic leptospiral strains and other bacteria were grouped as a non-target negative control, and RNase free water (Qiagen) was used as a no-target negative control.

TaqMan qPCR parameters and thermal cycling condition. A 20 µL reaction mixture was prepared for the qPCR containing 10 µL 2× SensiFAST Probe Hi-ROX Mix (Bioline), 0.8 µL (400nM) of each forward and reverse primer, 0.2 µL (100nM) of TaqMan probe and 8.2 µL of DNA template. The reaction was subjected to an initial denaturation step at 95°C for 5 min, then 40 cycles of 95°C for 10 s, and finally 60°C for 5 min. Positivity was determined by the cycle threshold (Ct) value.

The interpretations of Ct value were as follows: ≤29.00 denoted strong positive reactions, ≥30.00–≤37.00 positive reactions, and ≥38.00–≤40.00 weak reactions, which could represent infection states or environmental contamination (42). The assays were performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Sensitivity analysis. The sensitivity of the qPCR assay was determined. A fivefold serial dilution of *L. interrogans* DNA was used as a standard control and the estimated genome size of *L. interrogans* was 4,627,366 bp (31). The number of DNA copies was calculated based on the following formula: (DNA amount (ng) × 6.022×10^{23})/(length of template (bp) × $1 \times 10^9 \times 650$). The TaqMan qPCR was performed in triplicate, and DNA copies were averaged and recorded. The limit of detection (LOD) was determined based on the average amount from the triplicates of leptospiral DNA that could be detected. A standard curve was generated based on log DNA copies (*x* axis) and the mean Ct value (*y* axis). The qPCR amplification efficiency, E, was calculated using the formula $E = 10^{(-1/\text{slope})}$. The coefficient of regression (R^2) was also obtained from the standard curve to measure how well the regression predictions approximated the real data points (the closer R^2 to 1, the better the prediction).

Statistical analysis. Positive detections of conventional PCR and qPCR in whole blood and urine samples were recorded and analysed using descriptive statistics with 95.0% confidence intervals (CIs) using SPSS Statistics version 23 (IBM, Armonk, NY, USA). True positive leptospirosis and true negative leptospirosis groups were defined with the assumption that the conventional PCR was 100.0% sensitive and specific. The epidemiological sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the qPCR assays were calculated using MedCalc Statistical Software version 2014 (MedCalc Software, Ostend, Belgium), with which a detailed analysis for the whole blood and urine assays was also made.

Results

Serologic detection using microscopic agglutination tests. Of the 124 sera tested using the MAT, 53 dogs (42.7%; 95% CI: 34.0–51.4%) were seropositive with antibody titres ranging between 1:100 and 1:800. Multiple patients were seropositive for the Bataviae (n = 12), Javanica (n = 10), Icterohaemorrhagiae (n = 10), Australis (n = 3), Ballum (n = 3), Hardjobovis (n = 3), Malaysia (n = 3), and Pomona (n = 2) serovars. The least common leptospiral serovars observed based on seropositivity were Autumnalis, Canicola, Celledoni, Copenhageni, Cynopteri, Lai, and Pyrogenes (all n = 1). All dogs were seronegative for the Djasiman, Grippotyphosa, Hebdomadis, Patoc, and Tarassovi serovars.

Molecular detection using the conventional PCR. The total molecular detection rate of leptospiral infection using the PCR was 42.7% (53/124; 95% CI: 34.0–51.4%). Among the 53 positive dogs, 17 dogs were positive for blood only, 11 dogs were positive for urine only and 25 dogs were positive for blood and urine Altogether, the positive samples were obtained from 42 whole blood and 36 urine samples.

Primer and TaqMan probe specificity. Neither *LipL32*(1) nor *LipL32*(2) were able to detect intermediate or saprophytic leptospiral strains, nor to detect other bacteria strains. The *LipL32*(1) primer was able to detect all pathogenic leptospiral strains with Ct values ranging from 13.04 to 29.67. *LipL32*(2) was unable to detect three pathogenic leptospiral strains (Table 3) and showed a higher Ct value than *LipL32*(1). Therefore, *LipL32*(1) was selected for the detection of pathogenic *Leptospira* spp. in whole blood and urine samples in this study.

Sensitivity analysis. Analytical evaluation of the qPCR assay using *LipL32*(1) primers and respective TaqMan probe with standard control (DNA concentration 51.85 ng/μL) exhibited a linear relationship of Ct to the amount of leptospiral DNA, with a coefficient of regression (R^2) value of 0.997 (Fig. 1). The qPCR assay was able to amplify approximately five DNA copies per reaction in all replicates (Table 4). The amplification factor was two (equivalent to 99.9% efficiency). The lowest and highest Ct values generated from the standard curve were 12.47 and 32.00. Therefore, samples detected within the range of 12.47 to 32.00 were confirmed positive by qPCR.

Molecular detection using the qPCR. The total molecular detection rate of leptospiral infection using qPCR was 53.2% (66/124; 95% CI: 44.1–62.2%). Among the 66 positive dogs, 19 were positive for blood only, 5 were positive for urine only, and 42 were positive for blood and urine. Altogether, the positive samples were obtained from 61 whole blood and 47 urine samples.

Epidemiological sensitivity and specificity of the qPCR assay. Overall, the qPCR assay had a sensitivity of 100.00% (95% CI: 93.28–100.00%) and specificity of 81.69% (95% CI: 70.73–89.87%). Without reference to sample type, the PPV and NPV of the qPCR assay were 80.30% and 100.00% respectively, with an accuracy of 89.52%.

Detailed sensitivity and specificity of the qPCR assays for whole blood and urine. In total, 61 of the 124 (49.20%; 95% CI: 40.11–58.32%) whole blood and 47 of the 113 (41.60%; 95% CI: 32.40–51.24%) urine samples were detected as positive by qPCR. The Ct values for whole blood and urine samples ranged between 23.80 and 31.90 and 13.57 and 31.43, respectively. The qPCR assay for whole blood had a sensitivity of 100.00% (95% CI: 91.59–100.00%) and specificity of 76.80% (95% CI: 66.20–85.44%). The PPV and NPV of the blood assay were 68.90% and 100.00% respectively, with an accuracy of 84.70%. The qPCR assay for urine

had a sensitivity of 100.00 % (95% CI: 90.26–100.00%) and specificity of 85.70% (95% CI: 75.87–92.65%). The PPV and NPV of the urine assay were 76.60% and 100.00% respectively, with an accuracy of 90.30%.

Table 3. Comparison of Ct values between a qPCR assay with the *LipL32*(1) primers and one with the *LipL32*(2) primers

Group	DNA concentration (ng/µL)	*LipL32*(1) Ct value	*LipL32*(2) Ct value
Positive control			
L. borgpetersenii serovar Ballum strain Mus 127	26.4	15.33	32.08
L. borgpetersenii serovar Hardjobovis strain 117123	20.6	17.41	33.36
L. borgpetersenii serovar Javanica*	8.2	16.22	36.14
L. borgpetersenii serovar Javanica strain Veldrat Bataviae 46	41.0	14.35	33.96
L. borgpetersenii serovar Tarassovi strain Perepelitsin	16.1	20.54	36.39
L. interrogans serovar Australis*	9.9–11.9	15.43–15.58	33.78–34.38
L. interrogans serovar Australis strain Ballico	11.8	29.67	N
L. interrogans serovar Autumnalis strain Akiyami A	32.6	23.56	N
L. interrogans serovar Bataviae*	5.3–20.1	13.91–16.02	34.11–35.91
L. interrogans serovar Bataviae strain Swart	15.8	17.97	34.29
L. interrogans serovar Canicola strain Hond Utrecht IV	12.7	14.99	34.08
L. interrogans serovar Copenhageni strain M20	2.5	17.95	34.44
L. interrogans serovar Djasiman strain Djasiman	11.5	16.20	35.43
L. interrogans serovar Hebdomadis strain Hebdomadis	28.4	16.77	33.91
L. interrogans serovar Icterohaemorrhagiae strain RGA	16.8	14.3	32.81
L. interrogans serovar Lai strain Lai	12.8	19.22	36.19
L. interrogans serovar Pomona strain Pomona	7.2	18.05	35.44
L. interrogans serovar Pyrogenes strain Salinem	43.3	21.40	N
L. kirschneri serovar Cynopteri strain 3522C	29.7	14.44	33.60
L. kirschneri serovar Grippotyphosa strain Moskva V	42.3	13.04	31.79
L. kmetyi serovar Malaysia strain Bejo-ISO9	36.1	18.78	33.34
L. weilii serovar Celledoni strain Celledoni	13.0	24.15	40.52
Non-target negative control			
Bacillus spp.	9.9	N	N
Escherichia coli	16.0	N	N
L. biflexa serovar Patoc strain Patoc I	42.9	N	N
*L. selangorensis***	8.7–14.3	N	N
*L. wolffii***	6.7–19.3	N	N
Pseudomonas aeruginosa	84.9	N	N
Salmonella spp.	41.1	N	N
Staphylococcus aureus	14.4	N	N
Streptococcus spp.	4.2	N	N
No-target negative control			
RNase-free water	0	N	N

* – dog isolates; ** – environmental isolates; N – negative

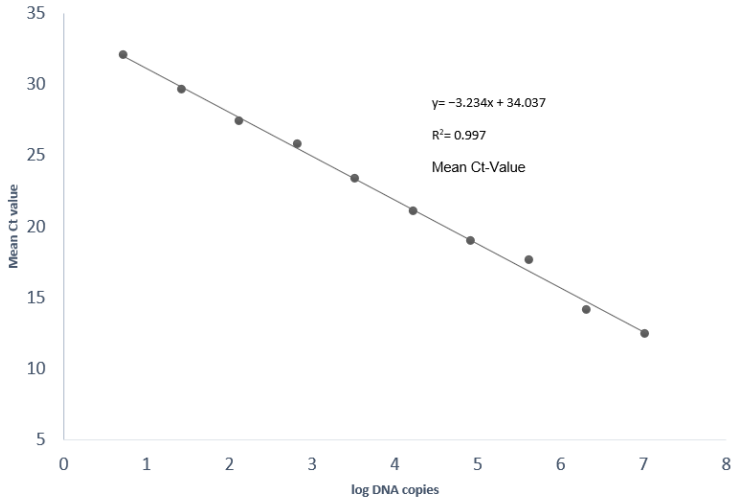

Fig. 1. Standard curves of the TaqMan probe–based qPCR assays showing amplification of successive fivefold dilutions of *Leptospira interrogans* genomic DNA. R^2 – coefficient of regression; Ct – cycle threshold

Table 4. Threshold cycle (Ct) values of fivefold serial dilution of *Leptospira* genomic DNA in a 20 µL reaction volume

Dilution factor	Mean DNA copy number/µL	log DNA copies	Mean Ct value
Standard control with DNA concentration of 51.85 ng/µL (primary stock)	10,382,320	7.01629441	12.47
1:5	2,076,464	6.317324406	14.15
1:25	415,292.8	5.618354402	17.66
1:125	83,058.56	4.919384397	18.96
1:625	16,611.712	4.220414393	21.05
1:3,125	3,322.3424	3.521444389	23.36
1:15,625	664.46848	2.822474384	25.79
1:78,125	132.893696	2.12350438	27.43
1:390,625	26.5787392	1.424534376	29.67
1:1,953,125	5.31574784	0.725564371	32.00

Discussion

In clinical diagnostic laboratories, real-time PCR methods (SYBR green and TaqMan chemistries) have gained popularity because they provide the opportunity for rapid diagnosis of leptospirosis in the first days of infection (8). To date, MAT remains the gold-standard method for diagnosis, but MAT and other serological tests are less useful if conducted during the acute phase of infection (6, 25). To improve the interpretation of the test, it is recommended to analyse paired serum samples taken at a two-week interval to confirm leptospirosis (35). In this study, 102 dogs presented with acute clinical illness, this being a phase lasting only up to one week, whereas 22 dogs were in the chronic phase. Therefore, molecular methods such as conventional and qPCRs were preferable to serological methods for the detection of the presence of *Leptospira* spp., especially in infected dogs in the acute phase. Nevertheless, the MAT results in this study remained important for serological evaluation (despite paired serum samples not having been collected) and the PCR results needed to be interpreted with those of the MAT to confirm leptospirosis. The molecular detection rates using the conventional PCR and the qPCRs were compared in the study.

Polymerase chain reactions allow early detection of *Leptospira* with high sensitivity and specificity (30). Routinely, conventional PCR has been adopted to diagnose canine leptospirosis locally (32). To date, several qPCR methods have been described in human leptospirosis, and the superior usefulness of the qPCR as a diagnostic tool was demonstrated over the conventional PCR (8, 29, 39), but the validity of this technique has not been determined. To our knowledge, this study was the first to evaluate the analytical performance of a TaqMan probe–based qPCR to detect *Leptospira* spp. using clinical samples from dogs diagnosed with kidney and/or liver disease.

The microscopic agglutination test is a sensitive assay, but because of the antigenic heterogeneity of *Leptospira* spp., the test requires many serovars as antigens (6). In this study, the overall serological detection of leptospiral infection in dogs with kidney and/or liver disease was 42.7%, which was much higher than the rate of previous local studies. The reason could likely be the specific selection of dogs to be recruited and the testing of the serum against the particular 20 leptospiral serovars selected. In comparison, one previous study investigated a larger population of apparently healthy shelter and working dogs (12), two investigated healthy dogs from a single location (16, 22) and one study was carried out among pet dogs (21). All of these studies only aimed to determine seropositivity among apparently healthy dogs using a panel of 10 leptospiral serovars, unlike in this study. The dogs recruited to this research were seropositive for 20 leptospiral serovars: Bataviae (n = 12), Javanica (n = 10), Icterohaemorrhagiae (n = 10), Australis (n = 3), Ballum (n = 3), Hardjobovis (n = 3), Malaysia (n = 3), and Pomona (n = 2) in more than one instance and for the other serovars in single instances.

Based on molecular detection with a conventional PCR, 42.7% of the dogs diagnosed with kidney and/or liver disease had leptospirosis. This result was consistent with a previous study which reported 42.4% (14/33; 95% CI: 25.6–59.3%) (28). Despite that study having a smaller sample size, the comparison is valid because the target population was similar. In contrast, a previous study reported lower molecular detection rates of 19.8% (26/131; 95%CI: 13.0–26.7%) (34) and 1.0% (1/106; 95% CI: 0.0–2.8%) (20). Despite the similarity between these studies in both having large sample sizes, this comparison must be regarded with caution because the target population recruited to that study were apparently healthy dogs. This could explain the lower detection rate reported.

This study used two qPCR assays, namely *LipL32(1)* and *LipL32(2)*, and both assays showed negative detection for other microorganisms, including intermediate and saprophytic *Leptospira* spp. that may have been present in the clinical samples and caused nonspecific amplification. Only the *LipL32(1)* assay could amplify all the pathogenic leptospiral strains; its average Ct value of 16.56 indicated strong positive reactions in which abundant target nucleic acid was detected in the positive control. The *LipL32(2)* assay differed in efficacy, producing an average Ct value of 30.94, indicating positive reactions and a moderate amount of target nucleic acid detected in the positive control. The *LipL32(1)* assay had higher sensitivity than

the *LipL32(2)* assay and therefore showed better performance in detecting pathogenic *Leptospira* spp. in the samples from the dogs in this study.

Analytically, the *LipL32(1)* assay amplified as few as approximately five DNA copies per reaction, which was close to the lowest theoretically possible LOD reported of three copies per reaction (7). The *LipL32(1)* assay LOD was comparable to previous molecular assays, which amplified between 1 and 10 copies per reaction (4, 5, 27, 29, 39). In terms of leptospiral coverage, another published assay that utilised a similar target showed the same results: it amplified all the pathogenic *Leptospira* spp. but did not amplify the intermediate and saprophytic *Leptospira* spp. (25).

The highest Ct value obtained from the standard curve of the *LipL32(1)* assay in this study was 32.00 and samples with higher values were considered negative. Theoretically, Ct values of 30.00 to 37.00 were positive reactions, but based on the analysis of the *LipL32(1)* assay's standard curve, any Ct value higher than 32.00 might be associated with a nonspecific reaction or contamination from the samples. To eliminate doubtful and false-positive results, only samples which yielded Ct values ≤32.00 were considered positive in this study.

The conventional PCR showed that 42 out of 124 whole blood and 36 out of 113 urine samples were positive for pathogenic *Leptospira* spp. Interestingly, the qPCR had a better detection rate, where 61 out of 124 whole blood and 47 out of 113 urine samples were positive for pathogenic *Leptospira* spp. Although the conventional PCR and the qPCR followed similar steps, they differ enough for many advantages of the qPCR to be discernible. Assays of the qPCR type can identify amplified fragments during the PCR process. A qPCR measures the amount of the product during the exponential phase, whereas a conventional PCR measures the product during the plateau phase. It was more effective to measure during the exponential phase because measurements taken during the plateau phase do not always clearly indicate the quantity of starting material. In the plateau phase of the amplification, depleted amounts of reagents are available for the amplification (some having been consumed during the exponential phase), and the amplification inhibitors are more active during this phase. Hence, accurate measurement is not possible in conventional PCR methods (14, 24). Moreover, a conventional PCR requires post-PCR analysis using agarose gel electrophoresis, which identifies the product either by size or sequence. Although running gel electrophoresis is relatively inexpensive, it is time-consuming and non-automated. It has low specificity since molecules of the same or similar weights cannot be easily differentiated (40, 43). This may explain why the qPCR assay used had better detection of positive reactions that were quantifiable from the samples compared to the conventional PCR.

Among the 53 seropositive dogs, 30 from which whole blood was drawn and 22 from which urine was collected were positive using the qPCR assay, whereas 16 dogs sampled by whole blood and 15 dogs sampled by urine were positive using the conventional PCR. This suggests that the dogs were in the convalescent phase, when antibodies might have started to react with the antigens, potentially reducing the amount of leptospires circulating in the body, but not to such an extent that they were undetectable using the molecular method. On this assumption, the qPCR had a better ability to detect infection during the convalescent phase.

The Ct values of the qPCR for whole blood ranged between 23.8 and 31.9 and those of the assay for urine samples were 13.57 to 31.43. Similarly, a previous study revealed that the canine whole blood samples had higher Ct values than urine samples (25). It was also mentioned that the sensitivity of the *LipL32* qPCR assay was 100 *Leptospira* spp./mL in whole blood samples, 1,000 *Leptospira* spp./mL in serum samples, and 10 *Leptospira* spp./mL in urine samples. The *LipL32* qPCR assay was able to detect low amounts of pathogenic *Leptospira* spp. in urine samples, resulting in low Ct values, which is consistent with the findings in this study (25). Therefore, whole blood and urine samples were preferable to serum samples for the detection of the pathogenic *Leptospira* spp.

The whole blood and urine samples tested using the qPCR assay were 100% detectable as positive when compared to these samples tested in the conventional PCR in this study, which means that the qPCR assay developed and validated in this research correctly identified all the positive dogs diagnosed with leptospirosis using PCR (it achieved 100% sensitivity). For specificity, the whole blood and urine samples tested using qPCR were 76.83% and 85.71% detected as negative, respectively when compared to these samples tested in the conventional PCR. The PPV of both whole blood and urine was >65.00%, which signifies that among dogs that had a positive qPCR assay result, the probability of disease was more than 65.00%. The NPV for both whole blood and urine samples was 100.00% which indicates that among dogs that had a negative qPCR assay result the probability of being disease-free was 100.00%. This showed that the qPCR assay used in this study was a good test, with accuracy of more than 80.00% for screening and diagnosis of acute canine leptospirosis, especially for dogs diagnosed with kidney and/or liver disease.

In conclusion, the *LipL32(1)* qPCR assay is a more reliable method for the detection of pathogenic *Leptospira* spp. than the *LipL32(2)* qPCR assay because the *LipL32(1)* assay was able to detect all pathogenic leptospiral strains with a low CT value. In addition, the *LipL32(1)* qPCR assay performed better in diagnosing acute canine leptospirosis than the conventional PCR, thus hastening an informed decision on therapeutic management and improving clinical outcomes. Although this study tested a targeted dog population, the high sensitivity and specificity of the *LipL32(1)* qPCR assay favour the exploration of this assay in a future study with a bigger population and a larger number of clinical samples.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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