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Uncovering latent infections in kidneys: A novel molecular approach for differential *Leptospira* detection

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ABSTRACT

Leptospirosis, a re-emerging zoonotic disease caused by *Leptospira* spp., poses significant global health and veterinary challenges. Long-term colonization of renal tubules by *Leptospira* in asymptomatic hosts highlights the need for sensitive detection methods. This study evaluates the chronic or latent *Leptospira* infections in kidneys using a novel molecular approach to examine individual immune responses differences. Digital PCR strategies employing newly developed primer-probe sets targeting the flagellar *fliG* gene were used to assess the presence of trace *Leptospira* in infected murine kidneys and urine samples from laboratory-confirmed leptospirosis patients. RNA-based digital PCR detected leptospires in 58 % (targeting *lipl32*) and 83 % (targeting *fliG*) of infected kidneys, demonstrating that the digital PCR strategy targeting the *fliG* gene offers superior sensitivity. Notably, the newly developed *fliG*-targeting assay detected as low as 20 fg of *Leptospira* DNA, offering ten-fold greater sensitivity than traditional qPCR for trace detection. This allows for differential detection of *Leptospira* species and facilitates monitoring of extremely low bacterial loads with greater sensitivity than conventional methods. We also observed regenerating renal tubules with mitosis and elevated cytokine expression in kidneys with transcriptionally active *Leptospira* during chronic infection. This approach aids in identifying latent infections and offers insights into individual variations. Our research provides a powerful molecular tool for epidemiological studies and public health surveillance, contributing valuable insights into the prevalence and transmission dynamics of this pervasive zoonotic disease.

1. Introduction

Leptospirosis caused by the genus *Leptospira* infection is the most widespread and neglected zoonotic disease which is also a waterborne disease prevalent in tropical and subtropical regions, accounting for over 70 % of cases occurring (Pappas et al., 2008; Vijayachari et al., 2008; Abela-Ridder et al., 2010; Costa et al., 2015; Karpagam and Ganesh, 2020). Climate change is leading to increased flooding events and a rise in the incidence of leptospirosis. Recent research has discovered that the disease is also associated with semi-arid regions and developed countries, such as the southwestern United States, may be related to the COVID-19 pandemic (Bini Viotti et al., 2020; Sanchez Fernandez et al., 2020; Sykes et al., 2022). Leptospirosis as a neglected burden at the human-animal-environment interface is a typical one health problem posing a public health threat and veterinary concern (Sykes et al., 2022).

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This infectious disease affects a broad spectrum of animals, including domestic animals as well as wildlife, such as mice, rats, horses, cows, pigs, dogs, sea lions and cats, and also humans (Hartskeerl and Terpstra, 1996; Cilia et al., 2020). Animal become infected through direct contact with urine contaminated with *Leptospira* or indirectly through contact with contaminated soil, water, or infected animals (Bradley and Lockaby, 2023). Animal leptospirosis has a broad range of clinical effects, ranging from mild, subclinical infection to multiple-organ failure and death. Subclinical and asymptomatic chronic variants are also the most common forms of animal leptospirosis (Di Azevedo and Lilenbaum, 2021). *Leptospira* are maintained and transmitted in nature through chronic kidney infections in carrier animals, which excrete the bacteria in their urine for many years (Bradley and Lockaby, 2023). In humans, clinical manifestations range from mild flu-like symptoms to severe multiorgan dysfunction, primarily affecting the kidneys, liver, and lung, while some patients are asymptomatic (Ganoza et al., 2010; Yang et al., 2015; Rajapakse, 2022; Rajaonarivelo et al., 2023). Leptospirosis poses a dual burden as it affects both animals and humans, with the animal burden being greater than human burden (Noguera et al., 2022). Therefore, control and prevention management for leptospirosis should prioritize animal populations. A recent study published in the Journal of Applied Ecology emphasizes mice as a significant yet underestimated source of leptospirosis, highlighting the persistent risk posed by these silent spreaders and the necessity of controlling the spread of the infectious disease (Marie Moinet, 2024). Mice are a common, asymptomatic reservoir of the disease, as a real sanitary threat of both human and animal leptospirosis (Marquez et al., 2019).

The kidney, where conditions in the renal tubules favor *Leptospira* survival, serves as the preferential site of infection. Pathogenic leptospires undergo colonization within the kidney to initiate immune responses, contributing to the development of leptospirosis kidney disease (Mc and Montgomery, 1952; Matsui et al., 2016; Silva et al., 2020; Chancharoenthana et al., 2022; Chou et al., 2023). These colonization leads to a persistent presence of leptospires in the kidneys (Ratet et al., 2014; Yang et al., 2015; Carrillo-Larco et al., 2019). Research suggests the persistent presence of leptospires in kidneys, potentially due to either latent infection or chronic active infection. The diagnosis of the silent chronic form of animal leptospirosis remains a challenge (Di Azevedo and Lilenbaum, 2021).

Due to the mild and asymptomatic presentation of leptospirosis, this disease is often overlooked. Considering the potential risk of renal disease associated with asymptomatic and chronic leptospiral infections, the utilization of highly sensitive diagnostic methodologies is crucial in addressing leptospirosis. Traditional detection methods such as visualization (direct dark-field microscopy examination), culture and serological techniques (microscopic agglutination test, enzyme-linked immunosorbent assay and Lepto dipstick assay) have been regarded as ineffective because of their low specificity and inadequate sensitivity (Goris and Hartskeerl, 2014; Niloofa et al., 2015; Koizumi, 2020; Martin et al., 2022). While the detection of acute leptospirosis through direct examination, serology and molecular-based techniques is relatively standardized, the diagnosis of chronic leptospirosis, particularly its subclinical form, remains challenging. Current detection methods for *Leptospira* infections have limited sensitivity and specificity, rendering them unable to detect low bacterial loads in asymptomatic carriers.

Molecular methods, including conventional PCR, real-time quantitative PCR (real-time qPCR) and loop-mediated isothermal amplification (LAMP), are emerging as crucial tools for detecting leptospiral DNA in clinical specimens. Housekeeping genes and pathogen-specific genes serve as typical targets, including 16S rRNA, lipoprotein and flagellar genes such as *rrs, lipl32, lfb1*, gyrB, and sec*Y*, for leptospiral detection (Gokmen et al., 2016; Rupak Nagraik, 2020; Di Azevedo and Lilenbaum, 2021; Bárbara Couto Roloff Padilha, 2022; Pinto et al., 2022; Rethinavelu et al., 2022). The *lipl32* gene, which encodes the major outer-membrane lipoprotein LipL32 found only in pathogenic *Leptospira* species, is currently the most commonly targeted gene for leptospiral detection (Podgorsek et al., 2020; Di Azevedo and Lilenbaum, 2021). Although both pathogenic and nonpathogenic *Leptospira* species can be detected for the target gene *rrs*, the gene is highly conserved across the bacterial kingdom, so false-positive results are likely to be detected.

Digital PCR has emerged as a reliable analytical chemistry method, providing greater sensitivity and precision in quantifying low-abundance targets (Salipante and Jerome, 2020; Chen et al., 2021; Lei et al., 2021). There is still lack of research on the application of digital PCR for detecting *Leptospira* species, especially in cases of latent or chronic infections. This study explores the utility of a digital PCR approach for enhanced sensitivity and precision in detecting low-abundance *Leptospira* targets, particularly in latent infections. Our findings provide evidence for the effectiveness of a digital PCR approach for the rapid diagnosis of trace amounts of *Leptospira* infection and its precision in distinguishing between distinct species of *Leptospira*.

This study focuses on developing a novel molecular approach, RNA-based digital PCR targeting *fliG* gene, to evaluate chronic or latent *Leptospira* infections in the kidneys, aiming to analyze differences in immune responses and their potential effects on disease progression among affected individuals. Despite the availability of various genetic markers for pathogenic *Leptospira* detection, such as *lipl32*, the *fliG* gene has not yet been explored as a target for molecular approach for *Leptospira* detection. The *fliG* gene encodes a protein associated with flagellar structures. Due to its conservation among *Leptospira* species, it is potentially marker for leptospiral detection. The combination of specific probes for distinguishing *Leptospira* species with the high sensitivity potential of digital PCR provides new targets for development of improved *Leptospira* detection methods. This highlights its potential utility in epidemiological surveillance and public health interventions aimed at managing and preventing *Leptospira* infections.

2. Materials and methods

2.1. Bacterial strains

Leptospira interrogans serovar Copenhageni Fiocruz L1–130 (ATCC number BAA-1198; a pathogenic species) and *Leptospira biflexa* serovar Patoc (ATCC number 23,582; a nonpathogenic species) were cultured at 28 °C under aerobic conditions in a medium consisting of 10 % *Leptospira* enrichment Elinghausen-McCullough-Johnson-Harris (EMJH) medium (BD Diagnostics) and 90 % *Leptospira* medium base EMJM medium. The number of motile leptospires was determined using dark-field microscopy with a Petroff-Hausser counting chamber (Schreier et al., 2009).

2.2. Chronic leptospiral infection in experimental mice

Seven- to eight-week-old C57BL/6 mice were intraperitoneally injected with 1×10^9 leptospires, while control mice were inoculated with EMJH media. Mice were randomized and allocated to the following groups: I) the control group (mice with base EMJH medium as a negative control; $n = 3$); II) the nonpathogenic *Leptospira* group (mice infected with *L. biflexa;* $n = 4$); and III) the pathogenic *Leptospira* group (mice infected with *L. interrogans;* $n = 12$). Mice were sacrificed at 7 and 28 days postinfection, after which the kidneys were harvested to extract genomic DNA and total RNA. All animal experiments were conducted under animal biosafety level 2 (ABSL-2) conditions and adhered to relevant guidelines for the use and handling of infected animals. The Institutional Animal Care and Use Committee of the Chang Gung Memorial Hospital in Taiwan approved all animal procedures and experimental protocols (Approval No 2,021,062,203 and No 2,021,121,801).

2.3. Genomic DNA and total RNA extraction

Genomic DNA was extracted from bacterial liquid cultures and whole kidney tissue homogenates using QIAamp DNA extraction kit following

the manufacturer's protocol (Cat. No 51,306; Qiagen, Hilden, Germany). Extraction of total RNA from whole kidney tissue homogenates was performed using RNA-BeeTM RNAzol reagent (Tel-Test, Friendswood, TX, USA) with DNaseI digestion according to the manufacturer's protocol. Subsequently, 1 μg of total RNA was reverse transcribed using the Transcriptor cDNA Synthesis kit (Cat. No 04,897,030,001; Roche Diagnostics, Germany). Quantification of both total DNA and RNA was assessed using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE), and the samples were stored at −20 °C until further analysis.

2.4. Molecular detection using PCR and real-time qPCR

PCR was performed with SuperRed PCR Master Mix (BIOTOOLS Co., Ltd., Taiwan) on a PTC-100 programmable thermal controller (M.J. Research Inc., Waltham, Massachusetts, USA). The resulting amplicons were visualized through 2 % gel electrophoresis. Real-time qPCR experiments were performed with TaqMan gene expression assays on an ABI ViiA7™ real-time PCR system (Applied Biosystems).

2.5. Selection of candidate genes and primer design

In order to identify new candidate gene markers for *Leptospira*, the whole-genome sequences of *L. interrogans* (NC_005823.1) and *L. biflexa* (CP000786.1) retrieved from the NCBI database were aligned using Blastn (Zhang et al., 2000) to identify shared and conserved genes across *Leptospira* species, with a focus on encoding membrane and flagellar proteins. The primers and probes were designed using the online PrimerQuest™ Tool on the Integrated DNA Technologies website (Integrated DNA Technologies Inc., USA) using the default criteria. The primer and probe details are shown in Table 1.

2.6. Digital PCR

Each reaction contained a Probe PCR Master Mix (Qiagen), primers, probes and cDNA template. The digital PCR was performed using the QIAcuity Digital PCR System (Qiagen) and QIAcuity Nanoplate (Qiagen), which are microfluidic dPCR plates that allow for the processing of up to 26,000 partitions/well (Dreo et al., 2014). The QIAcuity Software Suite (Qiagen, version 2.1.7) was used to determine sample thresholds using positive, negative, and no-template control wells with the manual global threshold approach based on the amplitude signal observed in negative control samples.

2.7. Kidney transcript analysis

Relative mRNA expression levels were determined on an ABI ViiA7 real-time PCR system (Applied Biosystems) and calculated using the comparative cycle threshold (Ct) method, with GAPDH serving as an endogenous control.

2.8. Histology

Mouse kidneys were formalin-fixed and paraffin-embedded for the hematoxylin and eosin (H&E) staining to evaluate inflammatory changes and kidney structural injury by light microscopy.

3. Results

3.1. Chronically Leptospira-infected mice: renal immune responses induced by leptospira spp. infection

To understand how *Leptospira* establish persistence or latency in reservoir host species, we utilized mice as an infection model. Mice serve as long-term carriers and transmitters of *Leptospira* spp., typically without developing disease (Richer et al., 2015; Gomes-Solecki et al., 2017; Hamond et al., 2022). Fig. 1A illustrates the strategic approach of infecting C57BL/6 mice with *Leptospira via* intraperitoneal injection. It highlights the subsequent changes in immune responses and bacterial colonization within the kidneys, following infection with either pathogenic or non-pathogenic *Leptospira* species. Fig. 1B shows that pathogenic *Leptospira* infection triggers an elevated immune response characterized by increased levels of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), following decreased anti-inflammatory cytokine (IL-10) levels over the 28-day infection period. This heightened inflammatory response, as evidenced by immune cell infiltration and bacterial colonization in renal tubules (Fig. 1C-F), can lead to chronic infection and tissue damage. In contrast, nonpathogenic *Leptospira* spp. infection also induces an immune response, as shown by elevated levels of TNF-α, IL-6, and IL-10 in the kidneys (Fig. 1B). Interestingly, the expression of the anti-inflammatory cytokine IL-10 appears to increase over time in nonpathogenic *Leptospira* infection, suggesting a potentially role in regulating the immune response. In addition, the H&E-stained tissue section also observed immune cell infiltration in kidneys infected with nonpathogenic *Leptospira* spp. (Fig. 1D).

Although immune responses are observed in kidneys infected with nonpathogenic *Leptospira*, it remains challenging to determine whether nonpathogenic *Leptospira* are present in trace amounts due to the lack of specific detection targets for nonpathogenic *Leptospira* spp. Current methods for detecting nonpathogenic *Leptospira* spp., such as 16S rRNA analysis, are prone to cross-reactivity with other bacteria, potentially leading to false-positive results and hindering accurate assessment of their presence in the kidneys. There is a need to develop more specific and sensitive detection methods for *Leptospira* that can simultaneously detect pathogenic and non-pathogenic *Leptospira* species for providing a more precise understanding of whether these bacteria establish latent or chronic (active form) infections in tissues.

Table 1
Primer-probe sets sequence designed for detection of *Leptospira* species.

Gene target	Primer/probe[a]	Sequence (5′ to 3′)[b]	Amplicon size (bp)[c]	*Leptospira* target	Reference
lipl32	*lipl32*_F	AAGCATTACCGCTTGTGGTG	242	*L. interrogans*	Matsui et al., 2017
	*lipl32*_R	GAACTCCCATTTCAGCGATT			
	*lipl32*_P	FAM-AAAGCCAGGACAAGCGCCG-BHQ1			
fliG	*fliG*_F	ATACTTCTGCGGGTGGTATTG	110	*L. interrogans; L. biflexa*	This study
	*fliG*_R	GCAAGTTCGGGATCTTCTTCT			
	*fliG*_P1	FAM-AGTTGAGATTCTAAACTTAGTCG ATCGGGGAACG-BHQ1		*L. interrogans*	
	*fliG*_P2	FAM-ACGGAGAAGACCATCATCGAAGCT-BHQ1		*L. biflexa*	

[a] (F), (R) and (P) indicate forward and reverse primer and probe sequences, respectively.
[b] the probe, labelled with a unique fluorescent reporter dye [6-carboxyfluorescein (FAM)] at the 5′-end and a 3′- Black Hole Quencher 1 (BHQ1), was designed to anneal to an internal sequence within the amplified region.
[c] bp, base pairs.

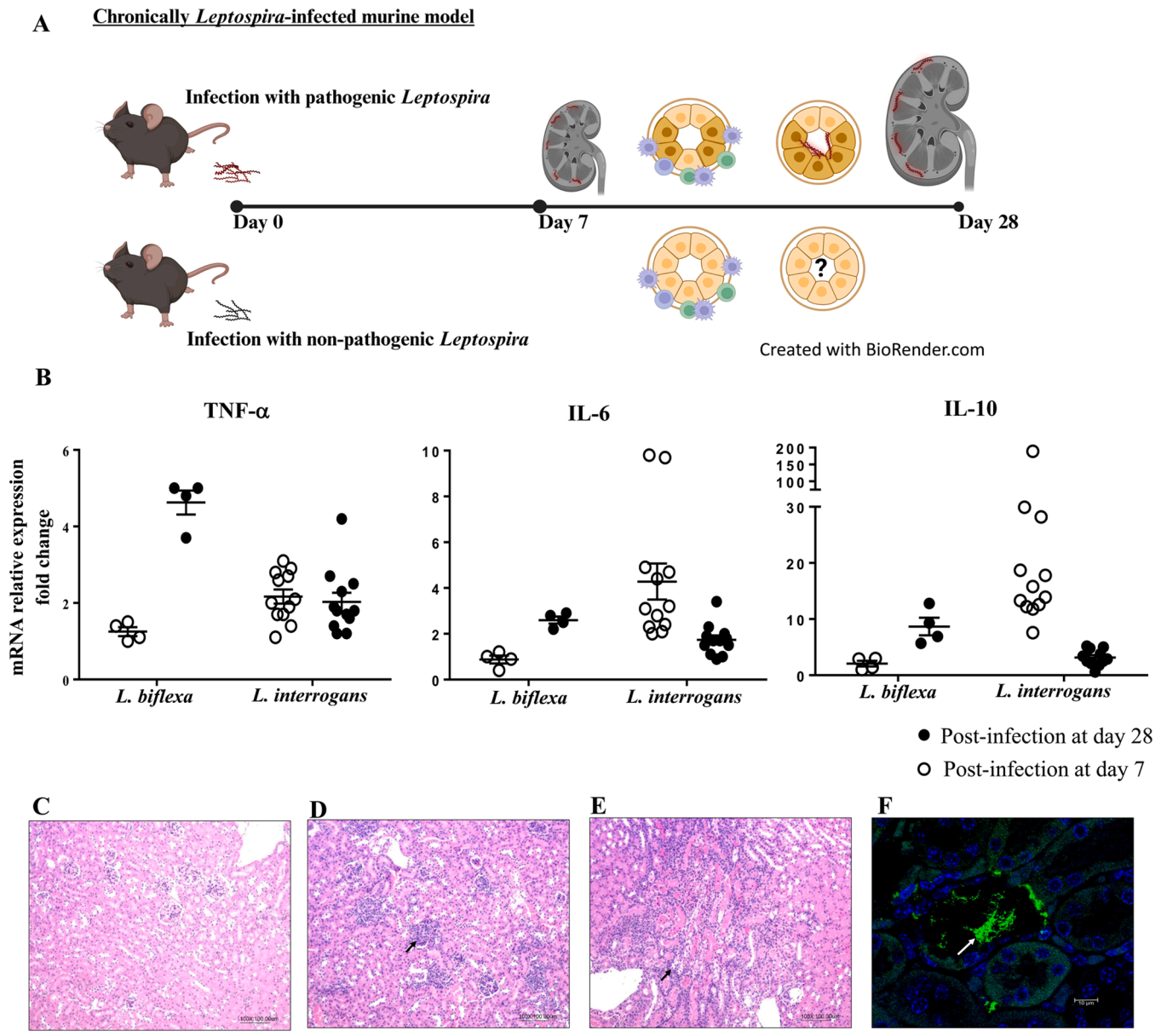


Fig. 1. Experimental design for investigating the impact of *Leptospira* infection on renal immune responses. (A) Schematic view of the study of immune responses and bacterial colonization in the kidneys following infection with either pathogenic or nonpathogenic *Leptospira* spp. **(B)** The immune responses to *Leptospira* infection are characterized by the mRNA expression of pro-inflammatory cytokines (TNF-α and IL-6) and the anti-inflammatory cytokine IL-10. Representative renal histological findings in uninfected **(C)** and infected **(D~F)** mice. These tissues were formalin-fixed and paraffin-embedded for histology and immunofluorescence study. Light microscopy of kidney tissues, stained with the hematoxylin and eosin (Magnification, 100 X). After infection at 28 days, inflammatory infiltration is observed in the nonpathogenic *L. biflexa*-infected **(D)** and pathogenic *L. interrogans*-infected **(E)** mice. **(F)** Bacterial adherence to the tubule lumen was found in the kidneys from mice infected with *L. interrogans* at 28 days post-infection. Confocal images of immunofluorescent staining (magnification of 400X). Anti-leptospiral LipL32 (green; white arrow) and DAPI staining (blue).

3.2. Comparative Leptospira detection in infected kidneys: advantages of RNA-based digital PCR

This study compared digital PCR (targeting the *lipl32* gene, which is unique to pathogenic *Leptospira* species) with conventional and real-time qPCR for detecting and quantifying leptospires in infected kidneys (Fig. 2). In our evaluation of digital PCR targeting the *lipl32* gene to detect pathogenic *Leptospira* in infected kidneys, we observed the following results at the DNA and RNA levels. Using digital PCR with RNA-based analysis, leptospires were detected in 58 % (7 out of 12) of infected mouse kidneys on both days 7 and 28. In contrast, DNA-based analysis using digital PCR identified leptospires in 55 % (6 out of 11) of infected kidneys on day 7 and in 42 % (5 out of 12) on day 28. When using real-time qPCR, RNA-based analysis detected leptospires in 0 % (0 out of 12) of infected kidneys on day 7 and in only 8 % (1 out of 12) on day 28 (Table 2). These findings reveal a statistically significant difference ($p < 0.05$, Wilcoxon Signed Rank Test) between DNA- and RNA-based analysis on day 28 using digital PCR, supporting the conclusion that RNA-based digital PCR provides superior sensitivity for detecting *Leptospira* in kidney tissues. Additionally, the RNA-based method offers greater sensitivity and reduces false positive results. However, it is notable that digital PCR targeting the *lipl32* gene did not detect bacteria in kidneys infected with nonpathogenic *Leptospira*, despite the presence of immune cell infiltration.

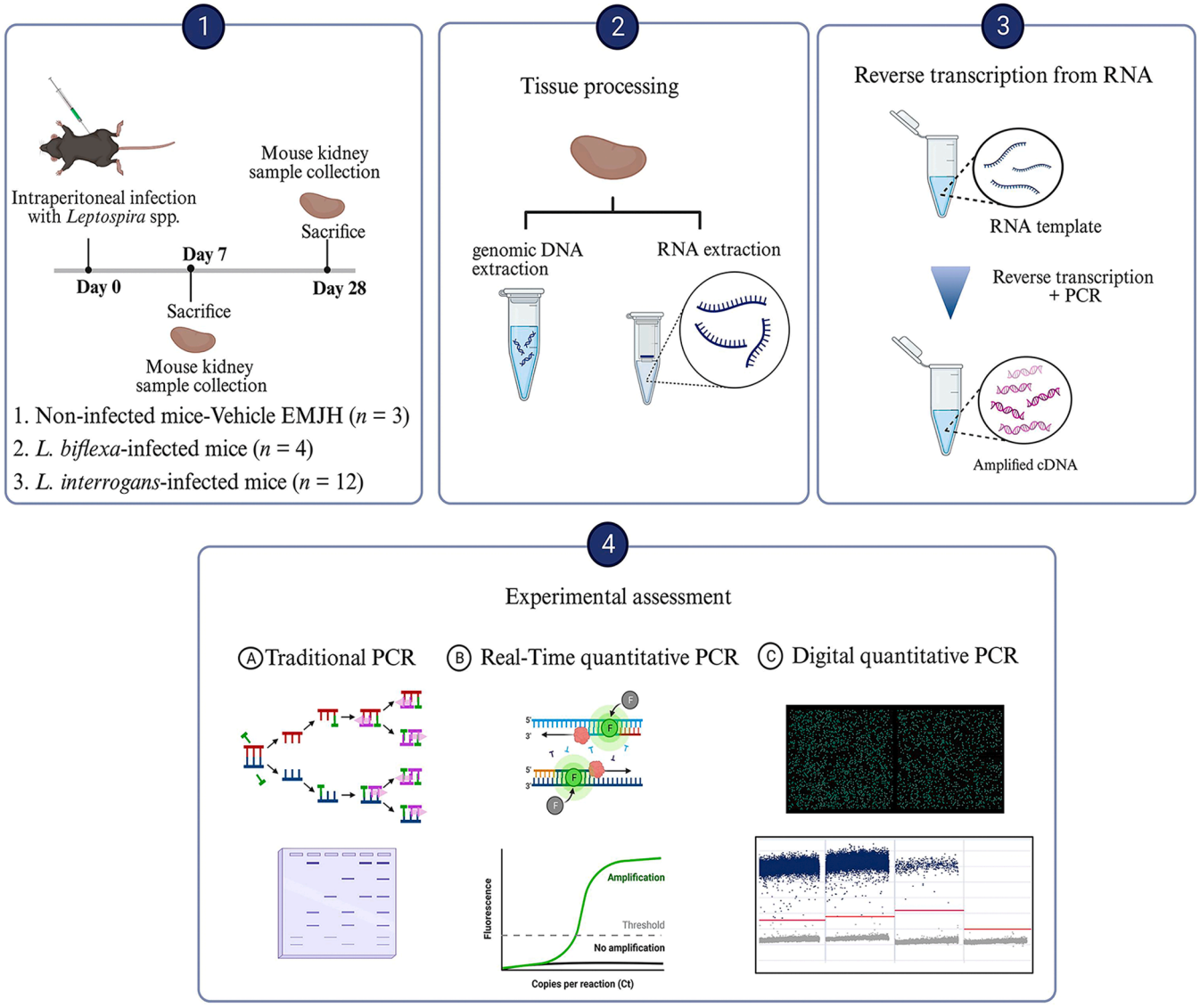


Fig. 2. The evaluation of digital PCR for the detection of pathogenic *Leptospira* infection in kidneys. Our study employed a digital PCR approach designed to target the *lipl32* gene, which is specific only to pathogenic species of *Leptospira*. This methodology, combined with conventional and real-time qPCR, was utilized for the detection and quantification of pathogenic leptospires in the kidneys of mice subjected to experimental *Leptospira* infection. Negative control mice were used as a reference for noninfected status, while mice with nonpathogenic *L. biflexa* infection served as an infection control group. Figure was created with biorender.com (accessed on Jan 2024).

Table 2
Proportion of infected mice assessed by examining the presence of *Leptospira* in the kidneys.

Target gene	Post-infection (days)	Groups	Genomic DNA extracted from kidneys			Total RNA extracted from kidneys		
			Traditional PCR[a]	Real-time PCR[a]	Digital PCR[a]	Traditional PCR[a]	Real-time PCR[a]	Digital PCR[a]
lipl32	7	Without infection	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)
		L. biflexa infection	0 (0/4)	0 (0/4)	0 (0/4)	0 (0/4)	0 (0/4)	0 (0/4)
		L. interrogans infection	0 (0/12)	8 (1/12)	55 (6/11)	0 (0/12)	0 (0/12)	58 (7/12)
	28	Without infection	0 (0/3)	67 (2/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)
		L. biflexa infection	0 (0/4)	50 (2/4)	0 (0/4)	0 (0/4)	0 (0/4)	0 (0/4)
		L. interrogans infection	0 (0/12)	33 (4/12)	42 (5/12)	50 (6/12)	8 (1/12)	58 (7/12)

[a] % of mice: Percentage (no. mice with the presence of *Leptospira* in the kidneys/no. total mice).

3.3. A newly developed digital PCR method for Leptospira detection: selection of Leptospira targets

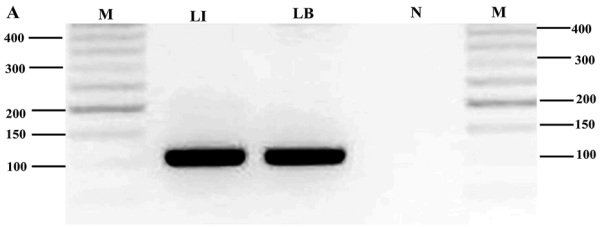
To identify novel *Leptospira* candidate gene markers, we designed new sets of primers and probes capable of simultaneously detecting and discriminating between pathogenic and nonpathogenic *Leptospira* species. The whole-genome sequences of *L. interrogans* and *L. biflexa* were downloaded from the NCBI database. A BLASTn search and sequence alignment were conducted to identify shared and conserved genes, particularly those encoding outer membrane, surface, and flagellar

proteins, between pathogenic and nonpathogenic *Leptospira* species (Zhang et al., 2000). Comparative sequence analysis of the *L. interrogans* and *L. biflexa* genomes using a BLASTn-based alignment method revealed 79 % identity between the nucleotide sequence of *fliG* (LIC10023), encoding a flagellar motor switch protein from *L. interrogans*, and that of *fliG2* (LEPBI_I3423) from *L. biflexa* (an alignment obtaining an E-value of zero). Alignment of the DNA sequence using the Martinez/Needlemen-Wunsch alignment method by MegAlign (DNASTAR, USA), with a gap penalty of 1.10 and a gap length penalty of 0.33, revealed that the sequence similarity index between LIC10023 and LEPBI_I3423 was 78.3 % (Fig. 3). Orthologous sequences to the selected nucleotide sequences were identified between *L. interrogans* and *L. biflexa* through whole-genome reciprocal best hit BLASTx analysis (Moreno-Hagelsieb and Latimer, 2008), indicating that protein sequences exhibiting >90 % identity and 99 % coverage were regarded as homologs. Hence, the orthologous gene pairs LIC10023 and LEPBI_I3423 between *L. interrogans* and *L. biflexa* were used for primer and probe design *via* the PrimerQuest™ Design Tool. We identified nucleotide sequence variant sites between the retrieved homologous sequences and then designed specific probes accordingly.

3.4. Validation of newly designed primer-probe sets for Leptospira species detection

The ability of these primers and probes to identify leptospiral sequences was initially evaluated using the BLASTn program against the NCBI sequence database. To further assess the utility of the novel primer pairs targeting the *fliG* gene developed in this study, we performed PCR analysis using the primer pairs and amplification procedure with genomic DNA from *L. interrogans* and *L. biflexa* (Fig. 4). The novel primer pairs (*fliG*_F and *fliG*_R) successfully yielded PCR products of the expected 110 bp size (Fig. 4A**)**, demonstrating their effectiveness in amplifying both pathogenic and nonpathogenic *Leptospira* species.

To confirm the specificity of novel probes targeting the *fliG* gene for the differential detection of *L. interrogans* and *L. biflexa*, we performed real-time qPCR analysis using novel primer pairs (*fliG*_F and *fliG*_R) and designed probes against genomic DNA preparations from *Leptospira* species. The specificity of the novel probes (*fliG*_P1 for *L. interrogans* and *fliG*_P2 for *L. biflexa*) was evaluated *via* real-time qPCR analysis. This analysis revealed proper amplification of the *fliG* gene from



B

primer-probe sets	Species name	Average Ct ± SD
*fliG*_F&R_P1	*L. interrogans*	18.3 ± 0.55
	L. biflexa	NA
*fliG*_F&R_P2	*L. interrogans*	NA
	L. biflexa	21.5 ± 0.21

Fig. 4. Validation of novel primer-probe sets for *Leptospira* species detection. (A) PCR amplification was performed using the primer pair *fliG*_F and *fliG*_R. Each reaction contained 25 ng of *Leptospira* genomic DNA under standard conditions. The amplification assay consisted of 30 cycles of 95 °C for 15 s, 60 °C for 75 s, and 72 °C for 75 s. Subsequently, the samples were maintained at 72 °C for 10 min, and the PCR products were separated via agarose gel electrophoresis and photographed. The genomic DNA from *L. interrogans* yielded a 110 bp fragment (Lane LI); genomic DNA from *L. biflexa* amplified a 110 bp fragment (Lane LB); negative control (water; Lane N); M, molecular size determined using a 50-bp ladder molecular marker. **(B)** RT−PCR amplification was performed using the primer pairs *fliG*_F and *fliG*_R combined with different probes designed in this study. Duplicate Ct values were averaged, and the standard deviation (SD) of the Ct values was calculated. NA: samples with no amplification detected.

L. interrogans using the *fliG*_P1 probe, with no amplification observed for *L. biflexa*. Similarly, proper amplification of *L. biflexa* was achieved using the *fliG*_P2 probe, while no amplification was detected for *L. interrogans* (Fig. 4B).

3.5. Evaluation of the sensitivities of Leptospira quantification and the clinical validation of digital PCR using novel primer-probe sets

Leptospira genomic DNA was serially diluted and analyzed using both

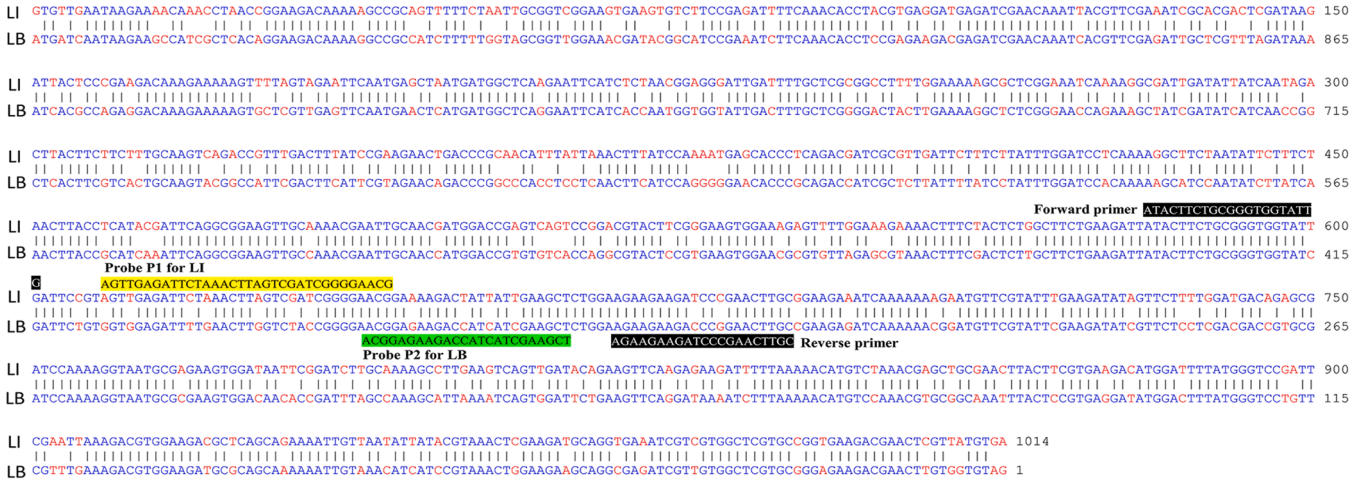


Fig. 3. Sequence alignment for each primer/probe pair using representative *Leptospira* species. Alignment of the DNA sequence of the *fliG* gene from *L. interrogans* serovar Copenhageni str. Fiocruz L1–130 (NCBI accession number: NC_005823.1) and the *fliG2* gene from *L. biflexa* serovar Patoc strain 'Patoc 1 (Paris)' (NCBI accession number: CP000786.1) was performed. Alignment of the DNA sequence using the Martinez/Needlemen-Wunsch alignment method by MegAlign (DNASTAR, USA) revealed that the sequence similarity index was 78.3 %. Identical nucleotides are shown as blue letters, and different nucleotides aligned at the same position are shown as red letters. The positions of the forward and reverse primers are indicated as white letters on a black background. The nucleotides corresponding to the probe for the target gene from *L. interrogans* (LI) are highlighted in yellow, while those for the target gene from *L. biflexa* (LB) are highlighted in green.

real-time qPCR and digital PCR to measure copy number. The accuracy of *Leptospira* DNA quantification by digital PCR was assessed by evaluating linearity within the detection range. This involved a tenfold serial dilution at concentrations ranging from 0.02 ng to 2 fg of *Leptospira* genomic DNA (for *L. interrogans*, the total genome copy input ranged from 4000 to 0.4; for *L. biflexa*, the total genome input of 4000 to 0.4 copy number estimates ranged from 4689.23 to 0.47). The copy number of *fliG* detected by digital PCR perfectly correlated (Pearson's correlation coefficient $r = 1.00$) with the theoretically calculated number of *Leptospira* copies, showing that *fliG* exhibited excellent linearity between the target input and measured values (Fig. 5). Notably, this method displayed greater sensitivity than real-time qPCR for quantitatively measuring leptospiral copy numbers, particularly at low concentrations (20 fg of input DNA; estimated copy number of 4.0) (Fig. 5). Real-time qPCR using the published primer-probe set targeting the *lipl32* gene detected as little as 200 fg of purified genomic DNA from *L. interrogans*. In comparison, our newly designed primers and probes targeting the *fliG* gene detected as little as 20 fg of purified genomic DNA from *L. interrogans*. Table 3 summarizes the results obtained from the newly developed primer pair and probe for evaluating the sensitivity of the digital PCR assays. These findings demonstrate that our novel primers and probes targeting the *fliG* gene offer superior sensitivity for *L. interrogans* detection compared to those targeting the *lipl32* gene. Additionally, digital PCR assays provide greater overall sensitivity for *Leptospira* detection than real-time qPCR assays.

To demonstrate the clinical application of the assays, we performed a confirmed patient diagnosed using the *Leptospira* IgG/IgM test kit. Urine samples collected before and after the initiation of antibiotic therapy were tested using digital PCR with our novel primer-probe sets (Fig. 6). The results showed that 71.8 copies per ml of *Leptospira* detected in the urine before antibiotic therapy and 0.1 copies per ml on day 10 after therapy, demonstrating that our strategy indeed effectively be used for non-invasive detection of *Leptospira* in urine specimens.

3.6. *Leptospira latent infection detection and the impact of long-term Leptospira spp. presence on renal tissue*

We performed an RNA-based assessment of *Leptospira* species identified and quantified in the kidneys of mice with chronic leptospiral infections using digital PCR coupled with newly designed primer-probe sets. In a digital PCR assay, leptospires targeting the *lipl32* and *fliG* genes were detected in 58 % (7 out of 12) and 83 % (10 out of 12), respectively, of the kidneys from infected mice on day 7 (Table 4). The *lipl32* transcript copy numbers detected in the kidneys of infected mice on day 7 ranged from 4.2 to 34.4 copies per μg of tissue, while the range of the *fliG* transcript copy numbers was 2.2 to 10.8 copies. In 41 % (5 out of 12) of the infected mice, the presence of the *lipl32* gene was not detected; however, very low levels of pathogenic *Leptospira* spp. were detected using a primer-probe set targeting the *fliG* gene (Table 4).

We examined the association between *Leptospira* quantity and cytokine expression levels in the kidneys of infected mice. Specifically, we investigated whether the bacterial load within the kidneys correlates with the expression levels of renal cytokines, such as IL-10, IL-6, and TNF-α, induced by infection. After *Leptospira* infection on day 7, we observed increased expression of IL-10, along with elevated IL-6 levels, in the kidneys of mice infected with pathogenic *Leptospira* spp. However, this pattern was not found in the kidneys of mice infected with nonpathogenic *Leptospira*. After nonpathogenic *Leptospira* infection on day 28, an increase in IL-10 expression was observed in the infected kidneys, along with elevated levels of IL-6 and TNF-α, whereas the presence of nonpathogenic *Leptospira* spp. was not detected in the infected kidneys (Table 4). After *Leptospira* infection on day 7, histopathological analysis revealed inflammatory cell infiltration in the kidneys of all mice infected with pathogenic *Leprospira* species, where leptospires were present. Furthermore, 50 % (6 out of 12) of the mice infected with pathogenic *Leptospira* species exhibited tubular degeneration and necrotic lesions. After *Leptospira* infection on day 28, the regeneration of renal tubules was observed in the infected kidneys, except for one mouse (#10 in Table 4), in which leptospires were not detected and cytokine expression remained normal.

As shown in **Supplementary Fig. 1**, histopathological examination of infected kidney sections revealed regenerating renal tubules accompanied by mitosis in kidneys where leptospiral presence (as detected by the *fliG* gene) and transcriptionally active leptospires (as detected by the *lipl32* gene) were detected, along with increased expression of cytokines (see #3 from mice infected with pathogenic *Leptospira* species in Table 4). This observation prompted a comprehensive analysis of cell cycle-related genes using the RNA-seq data previously generated for our investigation (GSE111249) (Chou et al., 2018). Pathway analysis revealed that cell cycle pathway pathways, including "Cell cycle checkpoints", "Mitotic Prometaphase", "Mitotic metaphase and anaphase" and "Mitotic G1 phase and G1/S transition" are involved in the effects of long-term *Leptospira* presence on renal tissue. Further studies are needed to understand how these genes contribute to leptospirosis kidney diseases.

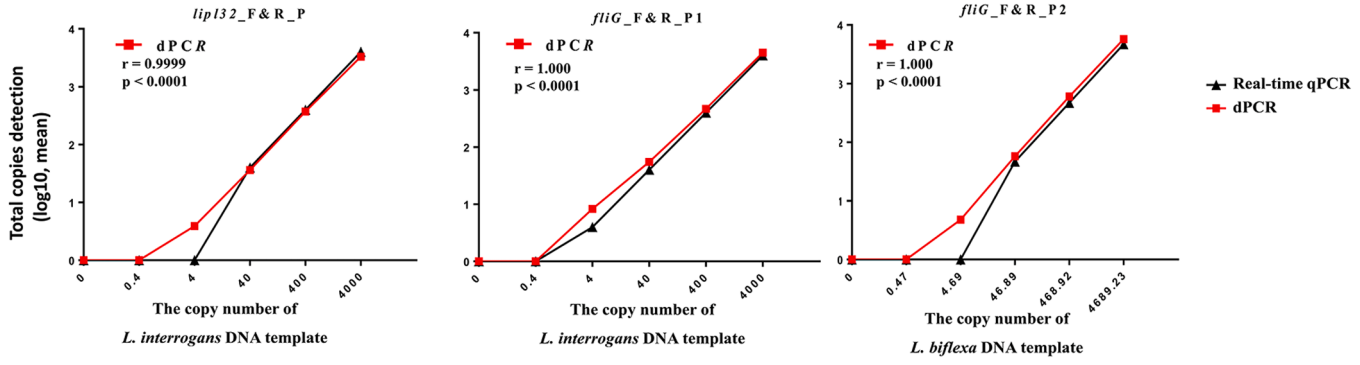


Fig. 5. Dynamic ranges and sensitivity for digital PCR assays targeting *lipl32* and *fliG* genes. The total copy number of each sample was determined using a digital PCR (absolute total copies detection; log values) and estimated from the Ct values obtained from real-time qPCR (data are presented as means; detailed values are provided in Table 3). The X-axis represents the 10-fold dilution series of *Leptospira* genomic DNA, and the numbers of copies were theoretically calculated using the copy number calculator tool on the Technology Networks server (https://www.technologynetworks.com/tn/tools/copynumbercalculator). Correlation analysis between the theoretically calculated *Leptospira* copies (X-axis) and the copy number measured in the dilution series using both real-time qPCR and digital PCR (Y-axis, displayed as the mean values; $n \geq 3$). Pearson's correlation coefficient (r) and p value are indicated. Statistical analyses were performed using Graph-Pad Prism 6.0 software (GraphPad Software, Inc.).

Table 3
The sensitivity evaluation of newly designed primers and probes using real-time qPCR and digital PCR.

Leptospira species primer-probe sets	*L. interrogans* *lipl32*_F&R_P				*L. interrogans* *fliG*_F&R_P1[b]				*L. biflexa* *fliG*_F&R_P2			
Assays	real-time qPCR		dPCR		real-time qPCR		dPCR		real-time qPCR		dPCR	
Quantity[a]	Ct mean ± SD[c]	RSD (%)[d]	Total copies mean ± SD[e]	RSD (%)[d]	Ct mean ± SD[c]	RSD (%)[d]	Total copies mean ± SD[e]	RSD (%)[d]	Ct mean ± SD[c]	RSD (%)[d]	Total copies mean ± SD[e]	RSD (%)[d]
20,000	29.53 ± 0.17	0.56	3,334.47 ± 110.85	3.32	28.99 ± 0.05	0.16	4,484.67 ± 163.12	3.64	31.45 ± 0.06	0.20	5,800 ± 448.54	7.73
2000	33.34 ± 0.08	0.24	372.50 ± 47.22	12.68	32.42 ± 0.15	0.46	464.47 ± 24.17	5.20	34.45 ± 0.78	2.27	602.20 ± 38.56	6.40
200	36.71 ± 0.45	1.21	35.92 ± 2.98	8.29	35.38 ± 0.23	0.66	55.07 ± 10.25	18.61	38.31 ± 0.30	0.79	57.88 ± 8.05	13.90
20	NA	NA	3.93 ± 0.85	19.68	39.37 ± 0.10	0.24	8.25 ± 0.37	4.47	NA	NA	4.74 ± 0.95	20.12
2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

[a] 10-fold serial dilution (from 0.02 ng to 2 fg) of genomic DNA extracted from *Leptospira* species.
[b] Developed in this study.
[c] the cycle threshold (Ct) values (Ct < 40, positive; otherwise, negative); Mean ± SD, all the Ct values were the average of the three experiments ± standard deviation; NA: samples with no amplification detected.
[d] RSD (%), relative standard deviation; used to analyze the precision of detection work, RSD (%) < 25 %, indicated this system has good repeatability.
[e] Total number of copies detected by digital PCR (dPCR); Mean ± SD, all the average of the six experiments ± standard deviation; NA: samples with no amplification detected.

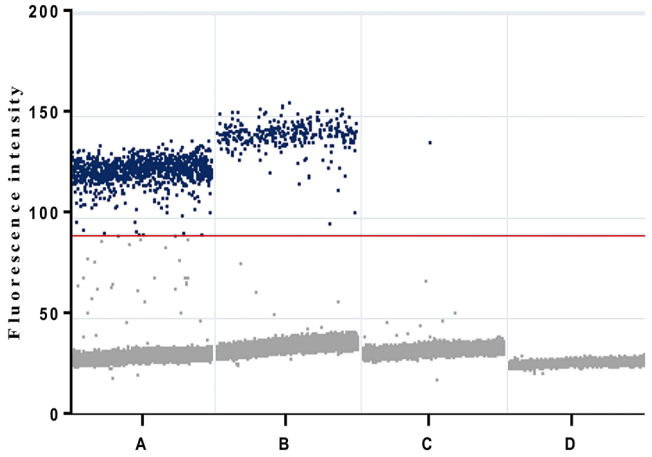


Fig. 6. Raw droplet scatter plot for the clinical validity of digital PCR assays targeting *fliG* genes in urine specimens from a laboratory-confirmed leptospirosis patient. Digital PCR for detection of *Leptospira* from A: positive control (cells infected with *L. interrogans*), B: urine specimens (before the initiation of antibiotic therapy), C: urine specimens (after the initiation of antibiotic therapy) and D: no template control. Positive droplets are obviously observed in the urine sample from before the initiation of antibiotic therapy. Blue dots represent the positive droplets, above the red horizontal threshold. Gray dots represent the negative droplets. The threshold for the detection of positive results was set at an amplitude of 90. Estimated copy numbers: Urine samples collected before the initiation of antibiotic therapy: 71.8 copy numbers per ml; Urine samples collected after the initiation of antibiotic therapy at day 10: 0.1 copy numbers per ml.

4. Discussion

This study introduces a novel digital PCR method for detecting active and latent *Leptospira* infections with enhanced sensitivity and specificity. The comparative analysis of *Leptospira* detection in infected kidneys demonstrates that digital PCR, both DNA- and RNA-based, is more effective than traditional and real-time qPCR methods, with the RNA-based approach offering superior efficacy over the DNA-based methods. This study provided insights into the dynamics of leptospiral presence in infected kidneys over time. Notably, our results revealed that leptospires remained detectable by detecting the *lipl32* gene in the kidneys of more than half of the infected mice even at 28 days after infection, highlighting the chronic infection of the pathogen in renal tissues. This finding emphasizes the importance of continued monitoring and timely intervention in chronic leptospirosis kidney disease.

We further developed novel primer-probe sets targeting the *fliG* gene for *Leptospira* detection, which exhibited heightened sensitivity in comparison to the conventional *lipl32* gene. Through PCR and real-time qPCR analyses, we confirmed the ability of these primer-probe sets to detect and discriminate between *L. interrogans* and *L. biflexa*. By employing tenfold dilutions of *Leptospira* genomic DNA to test novel primer-probe sets targeting the *fliG* gene, the results demonstrated high sensitivity, highlighting its superior performance in detecting *L. interrogans*. The integration of a digital PCR approach with our newly developed *fliG*-targeting assay enables simultaneous detection and differentiation of pathogenic and nonpathogenic *Leptospira* species, representing a valuable tool for *Leptospira* detection.

The quantification of transcript copy numbers revealed the dynamic range of *Leptospira* abundance in infected kidneys. Notably, in a subset of infected mice in which the *lipl32* gene was not detected, the *fliG* gene-targeted primer-probe set successfully identified very low levels of *Leptospira* spp. Our findings challenge the previously notion of rapid clearance of nonpathogenic *Leptospira* in living host (Surdel et al., 2022). As identified by the novel primers and probes targeting the *fliG* gene, only one out of four mice had detectible levels of nonpathogenic *Leptospira* at 7 days postinfection by the novel primers and probes targeting the *fliG* gene. This finding suggests that nonpathogenic *Leptospira* may persistent in kidney tissues for 7 days than previously thought, highlighting the importance of sensitive detection methods. Moreover, the differential detection patterns observed between the *lipl32* and *fliG* genes highlight the importance of utilizing multiple target genes for comprehensive pathogenic *Leptospira* detection. Interestingly, at 28 days postinfection, trace amounts of *fliG* gene expression were detected in the kidneys of some mice infected with pathogenic *Leptospira*, while *lipl32* gene expression was undetectable. This suggests the potential presence of latent infection in leptospirosis kidney disease. These findings emphasize the importance of longitudinal monitoring of leptospiral infections and the dynamic interplay between pathogen clearance and persistence in host tissues. The precise quantification of leptospiral abundance in infected tissues provides important information about disease progression, host-pathogen dynamics, and the efficacy of

Table 4
Quantification of leptospires and cytokine expression in the kidneys of infected mice. We employed a digital PCR approach specifically targeting the *lipl32* gene (for detecting pathogenic *Leptospira* spp.) and the *fliG* gene (for differential detection of pathogenic and nonpathogenic *Leptospira* spp.) to assess the number of leptospires in kidneys. To evaluate the effect of the *Leptospira* presence on cytokine expression in infected mice, we quantified the expression levels of cytokines, IL-10, IL-6 and TNF-α, using a real-time qPCR assay.

After infection at day 7		Without infection			Nonpathogenic *L. biflexa* infection				Pathogenic *L. interrogans* infection									
		#1	#2	#3	#1	#2	#3	#4	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12
lipl32	Concentration	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.3	15.2	0.0	0.0	0.0	4.2	6.6	34.4	0.0	6.7	9.8	0.0
fliG	(copies/ μg RNA)	0.0	0.0	0.0	0.0	0.0	0.0	2.1	8.4	2.2	8.8	4.4	4.4	6.6	8.7	0.0	12.9	0.0	6.5	4.4
IL-10[a]	Expression level	1.0	1.0	1.0	1.4	1.0	2.9	3.0	13.9	17.8	7.6	12.2	12.6	28.2	18.7	15.8	189.0	29.9	13.3	11.8
IL-6[a]	(Fold change)	1.0	1.0	1.0	0.4	0.9	1.2	1.0	4.4	3.2	2.4	2.0	2.1	4.7	9.8	4.9	2.3	9.7	3.1	2.8
TNF-α[a]		1.0	1.0	1.0	1.0	1.4	1.5	1.1	2.6	1.7	1.4	1.7	2.1	3.1	2.7	2.0	2.8	2.9	1.9	1.1
After infection at day 28		Without infection			Nonpathogenic *L. biflexa* infection				Pathogenic *L. interrogans* infection									
		#1	#2	#3	#1	#2	#3	#4	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12
lipl32	Concentration	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1680.4	77.8	135.1	597.2	49.3	13.3	0.0	0.0	0.0	0.0	0.0	285.8
fliG	(copies/ μg RNA)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.2	2.2	2.2	2.2	4.4	4.4	2.2	2.1	0.0	0.0	2.2	6.5
IL-10[a]	Expression level	1.0	1.0	1.0	6.9	5.7	12.8	9.3	2.9	0.6	3.4	2.5	5.0	5.2	3.8	2.5	2.1	0.6	4.8	1.8
IL-6[a]	(Fold change)	1.0	1.0	1.0	2.5	2.2	2.8	2.9	1.7	1.1	2.3	1.9	3.4	1.8	1.5	1.5	1.0	0.9	1.7	2.0
TNF-α[a]		1.0	1.0	1.0	5.0	3.7	5.0	4.8	1.8	1.2	2.3	1.8	4.2	2.5	1.9	1.7	1.2	1.6	2.7	1.4

[a] TaqMan probes were used for the indicated genes: Mm00439614_m1 (interleukin (IL)−10), Mm01210733_m1 (IL-6), Mm99999068_m1 (TNF-α) and Mm99999915_g1 (GAPDH).

therapeutic interventions.

Our previous studies revealed global alterations in renal gene expression in response to leptospiral infection, revealing that leptospires induced immune-related pathways within damaged kidneys (Chou et al., 2018, 2021, 2023). Histopathological examination of kidneys with detectable leptospiral presence and active transcriptional activity revealed regenerating renal tubules accompanied by mitosis, indicative of ongoing tissue repair processes. Elevated cytokine expression suggests interplay between the renal milieu, tissue injury, and leptospiral infection.

5. Conclusions

The *fliG*-targeting digital PCR assay significantly improves the detection sensitivity of latent *Leptospira* infections in kidney tissues, demonstrating superior performance compared to conventional qPCR methods. Our research emphasizes a novel molecular approach for the differential detection and tracking of small quantities of *Leptospira* spp., aiding in the exploration of low bacterial burdens in asymptomatic carriers. This approach provides a deeper understanding of latent and chronic *Leptospia* infections, addressing a critical public health issue and ultimately aiding in the control of this pervasive zoonotic disease. While this study demonstrates the development of a potentially useful digital PCR assay for detecting *Leptospira*, a limitation is that the assay has not yet been tested on a large number of clinical specimens or isolates from epidemiological studies. Future research should focus on evaluating this method across a broader range of clinical isolates to confirm its broader applicability, providing valuable insights into the prevalence and transmission dynamics of leptospirosis.

Supplementary material

Figure S1. Histopathologic examination of kidney tissues with the presence of transcriptionally active leptospires. (A) Inflammatory cell infiltration observed in the interstitium. **(B)** Renal tubule regeneration observed in several adjacent renal tubules (indicated by white arrows) with mitosis observed (highlighted by yellow arrowhead). (Representative section from mouse #3 infected with pathogenic *Leptospira* species, as indicated in Table 4) (hematoxylin and eosin staining, original magnification ×400).

Declaration of generative AI and AI-assisted technologies in the writing process

Quillbot was used to check grammar and spelling in order to improve readability. After using this tool, the authors reviewed and edited the content and take full responsibility for the content of the publication. Part of the figures were created by BioRender program (www.biorender.com).

Ethical approval statement

This study was approved by the Medical Ethics Committee of Chang Gung Memorial Hospital (approval number: 202301580B0). Patients who tested serologically positive for leptospirosis and provided informed written consent to participate in the study were included for evaluation.

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CRediT authorship contribution statement

Li-Fang Chou: Conceptualization, Funding acquisition, Project administration, Writing – original draft. **Yi-Chun Liu:** Formal analysis, Supervision, Writing – review & editing. **Huang-Yu Yang:** Investigation, Resources. **Ya-Chung Tian:** Resources, Supervision. **Chih-Ho Lai:** Formal analysis, Supervision. **Ming-Yang Chang:** Resources, Formal analysis. **Cheng-Chieh Hung:** Software, Validation. **Tong-Hong Wang:** Methodology, Software. **Shen-Hsing Hsu:** Methodology, Data curation. **Chung-Ying Tsai:** Data curation, Visualization. **Pei-Yu Hung:** Methodology, Validation. **Chih-Wei Yang:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

L.-F. C., P.-Y. H. and C.-W. Y. are inventors (Kidney Research Center, Chang Gung Memorial Hospital) on a patent for "Innovative method for nucleic acid detection tracking minute quantities of Leptospira spp. in biological tissues and environmental samples" (US Provisional Patent Application 63/605,750, filed 4 December 2023). The remaining authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2024.100327.

Data availability

I have shared our data at the attached file step

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