TROPICAL LIFE SCIENCES RESEARCH

Evaluation of Novel Primers for Rapid Single-Step Detection of Pathogenic *Leptospira* spp. Using Duplex Polymerase Chain Reaction (PCR) Assay

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Highlights

- New *rrs*/*lipL32* primer sets enable duplex detection and differentiation of pathogenic Leptospira.
- Duplex PCR achieved 100% sensitivity and specificity, outperforming *lfb1*/*secY* singleplex assays.
- Limit of detection: 100 cells/mL in urine and 30 fg/µL gDNA, supporting early clinical diagnosis.

EARLY VIEW

Evaluation of Novel Primers for Rapid Single-Step Detection of Pathogenic *Leptospira* spp. Using Duplex Polymerase Chain Reaction (PCR) Assay

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Running title: Novel Primers for Duplex PCR of Leptospira spp.

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Abstract: Pathogenic *Leptospira* spp. infections are the cause of the endemic disease leptospirosis. The flu-like and febrile symptoms can deteriorate severely and become fatal if not treated with antibiotic therapy on time. However, the current gold standard diagnostic method being used, the microscopic agglutination test (MAT), can only detect antibodies approximately one week after the onset of symptoms, besides, it requires skilled personnel and live cultures. In this study, two primer sets targeting *rrs* and *lipL32* genes were designed for simple, multiplex, and rapid molecular detection of *Leptospira* spp. The duplex PCR assay was evaluated using 15 *Leptospira* strains, comprising both pathogenic and non-pathogenic species, and demonstrated simultaneous detection and clear differentiation of pathogenic *Leptospira* spp. within a single PCR run. In a duplex PCR setting, the assay showed 100% sensitivity for both urine-spiked *Leptospira interrogans* serovar Copenhagenii and its gDNA, with a detection limit of 10^0 bacteria/mL for urine and 30 fg/uL for gDNA samples. The results were comparable to the adopted primers targeting *lfb1* or *secYIV* genes in a singleplex PCR setting. The duplex PCR assay showed 100% specificity in detecting *Leptospira* spp. by amplifying the highly conserved *rrs* gene in *Leptospira* spp. and pathogenicity differentiation through the highly conserved lipoprotein encoding *lipL32* gene in pathogenic strains. In contrast, the specificity of the singleplex PCR assay targeting *lfb1* or *secYIV* genes for pathogenic strain differentiation was 89% and 78%, respectively.

Keywords: *Leptospira* sp., duplex PCR (dPCR), *rrs*, *lipL32*, diagnostics

INTRODUCTION

Leptospirosis is a worldwide zoonotic and neglected infectious disease caused by pathogenic bacteria of the *Leptospira* genus from the family *Leptospiraceae* (Vinetz 2001). To date, twenty-three species of *Leptospira* have been described and classified into three groups based on their pathogenicity, i.e., pathogenic, intermediately pathogenic, and non-pathogenic natures, with more than 24 serogroups and 300 serovars recognized (Garcia-Lopez *et al.* 2023; Ali. *et al.* 2018; Pérez *et al.* 2020). Leptospirosis is endemic in several developing countries, such as Malaysia, Sri Lanka, Thailand, and Brazil, with outbreaks exacerbated by rainy seasons and floods (Soo *et al.* 2020; Vincent *et al.* 2019). Globally, an estimated one million human leptospirosis cases occur each year, resulting in approximately 60,000 deaths (Rajapakse *et al.* 2025). Economically, leptospirosis has been estimated to cause a global productivity loss of Int$29.3 billion, with upper estimates reaching Int$52.3 billion, and the greatest burden observed in the Asia–Pacific region (Agampodi *et al.* 2023). Leptospirosis is often neglected due to its mild early symptoms that are similar to many other febrile illnesses, such as influenza and COVID-19. Delayed diagnosis is the typical cause of its fatality, even

though it is curable with antibiotics (Haake & Levett 2015). Consequently, severe leptospirosis can lead to hepatitis, haemorrhage, renal failure, and even death. Rapid and robust diagnostic methods for leptospirosis are therefore crucial to reduce its morbidity and mortality rate.

Leptospirosis is diagnosed by either detecting the presence of the antigen or the pathogen or detecting the antibodies produced by the patient toward the pathogen. The latter type is known as the microscopic agglutination test (MAT) and is currently used as the 'gold standard' for leptospirosis diagnosis. MAT identifies the serovars of *Leptospira* spp. by determining the strain that causes the antibodies isolated from the patient to agglutinate (Picardeau *et al.* 2014). Despite its capability to identify the circulating serovars in a region, MAT is challenged by the lack of skilled personnel and the high requirement for laboratory facilities to maintain a wide range of live *Leptospira* cultures. To date, there are more than 250 antigenically distinctive serovars of *Leptospira* spp. identified globally (Haake *et al.* 2015; Hagedoorn *et al.* 2024). Furthermore, MAT displays a lower sensitivity compared to molecular diagnosis methods, where the antibodies can only be detected after one week of infection, leading to delayed medical intervention (Boonsilp *et al.* 2011; Goris & Hartskeerl 2014; Jaiswal *et al.* 2019). Consequently, MAT and other serological tests are not practically applicable for rapid diagnosis during the acute phase of infection (Smythe *et al.* 2009).

On the other hand, molecular diagnosis methods offer high sensitivity and accuracy by detecting nucleic acid of the pathogen. Its high utility was recently demonstrated during the COVID-19 pandemic, where PCR excelled in detecting the presence of the virus. Its wide employment is encouraged by great advancement in PCR and gene sequencing technologies in the past decade, allowing for more accurate primer design from updated gene databases. Besides being able to detect small amounts of viruses or bacteria in clinical samples, PCR-based methods are not affected by previous infections or vaccinations, i.e., cases where the patients may be exhibiting antibodies toward environmental or previous infections by *Leptospira* spp.

Leptospirosis is recognised by WHO as a priority zoonotic disease requiring a One Health approach that integrates human, animal, and environmental sectors for effective surveillance and response (WHO, 2010; WHO, 2020). This is especially critical in tropical regions where rainfall, flooding, and rodent reservoirs drive frequent outbreaks. The development of molecular tools for rapid early detection of pathogenic *Leptospira* strains can support these One Health goals by improving frontline diagnostic capacity and enabling timely public health intervention.

In this study, two novel specific primer sets targeting the *rrs* and *lipL32* genes were designed. The *rrs* gene is a highly conserved gene encoding for the 16S ribosomal RNA (rRNA) in the bacterial kingdom (Landolt *et al.* 2023; Ali *et al.* 2018). On the other hand, the *lipL32* gene is a robust target gene for the detection of pathogenic *Leptospira* spp., which

encodes a major outer membrane lipoprotein that is highly conserved among pathogenic *Leptospira* spp. (Natarajan *et al.* 2023). For enhanced detection efficiency, a duplex PCR was set up for simultaneous detection of *Leptospira* spp. and differentiation of pathogenic *Leptospira* spp. against the non-pathogenic strains. As a comparison of the sensitivity and specificity in differentiating pathogenic *Leptospira* spp., two previously reported primer sets targeting the *lfb1* gene (encoding a protein of unknown function) and the *secY* gene (encoding the preprotein translocase) were used, and the evaluation was conducted in a singleplex setting (Yeoh *et al.* 2023). The highly virulent *Leptospira interrogans* serovar Copenhagenii was used for sensitivity evaluation. The urine-spiked bacterial sample was also used for sensitivity evaluation to report the potential of urine samples for *Leptospira* spp. detection. For specificity evaluation, intermediately pathogenic *Leptospira* spp. were also included besides pathogenic and non-pathogenic strains, due to the reports of human febrile symptoms caused by several species of these 'pathogenic group II' *Leptospira* spp. (Piredda *et al.* 2021; Puche *et al.* 2018; Vincent *et al.* 2019).

Although the *rrs* and *lipL32* genes are among the most common molecular targets for the detection of pathogenic *Leptospir*a spp. (Levett, 2001; Boonsilp et al., 2011; Ahmed et al., 2009), many earlier primer–probe sets were developed exclusively for singleplex PCR formats. Such assays may display variable analytical performance across sample types and strains, leading to reduced sensitivity or inconsistent specificity (Stoddard et al., 2009; Bourhy et al., 2011; Podgoršek et al., 2020). In addition, the expanding genetic diversity of pathogenic *Leptospira* circulating across different geographic regions can influence sequence conservation within commonly targeted loci. Thus, periodic reassessment of primer and probe design is necessary to maintain assay coverage and binding efficiency (Smythe et al., 2002; Pérez et al., 2020; Bourhy et al., 2011). Therefore, developing a duplex PCR assay and designing updated primers targeting the *rrs* and *lipL32* genes with optimized specificity represent continuous efforts to improve assay compatibility, diagnostic accuracy, and overall performance of *Leptospira* detection.

MATERIALS AND METHODS

Bacterial strains and cultivation

A total of 15 *Leptospira* strains obtained from Biodefense and Emerging Infections Research Resources Repository (BEI Resources) and American Type Culture Collection (ATCC) were used for the primers' specificity assessment, as well as *Escherichia coli* DH5α, *Bacillus* sp., and *Staphylococcus aureus* from the Centre for Chemical Biology Microbial Biodiversity Library (CCB MBL) (Table 1). *Leptospira* strains were maintained in the Ellinghausen-McCullough-Johnson-Harris (EMJH) media (BD Diagnostics, Sparks, MD) at 30 °C.

Subsequent subcultures in the liquid EMJH medium were maintained at the same temperature for 7 days. Non-leptospiral bacterial strains were retrieved from storage in 20% glycerol at −20 °C. The bacteria were grown overnight in lysogeny broth (LB) at 37 °C with shaking at 200 rpm prior to genomic DNA (gDNA) extraction. The identity of the bacteria was confirmed using standard biochemical methods and partial 16S rRNA sequencing.

Table 1: Bacteria used in specificity evaluation of the duplex PCR assay.

Organisms	**Sources**	**Duplex PCR**		**Singleplex PCR**	
		LipI32	**Rrs**	**LFB1**	**SecYIV**
Pathogenic					
Leptospira interrogans serovar Copenhageni strain Fiocruz L1-130	ATCC BAA-1198D-5	+	+	+	+
L. interrogans -- 2006006971 (Serovar Grippotyphosa)	BEI Resources NR-19434	+	+	+	+
L. interrogans -- L495 (Serovar Manilae)	BEI Resources NR-19816	+	+	+	+
Leptospira interrogans -- HAI0024 (Serovar Icterohaemorrhagiae/Copenhageni)	BEI Resources NR-19892	+	+	+	+
L. interrogans -- Verdun LP (Serovar Icterohaemorrhagiae)	BEI Resources NR-19928	+	+	+	+
Leptospira kirschneri -- 1051 (Serovar Bim)	BEI Resources NR- 22287	+	+	+	+
L. interrogans -- CSL4002 (Serovar Pomona)	BEI Resources NR- 35358	+	+	+	−
Leptospira kmetyi strain Bejo-Iso9T (serovar Malaysia)	BEI Resources NR- 22254	+	+	+	+
Leptospira noguchii CZ 214T (Serovar Panama)	BEI Resources NR-22283	+	+	−	−
Intermediately pathogenic					
Leptospira hovindhougenii -- B5-022 (Serovar Osterbro)	BEI Resources NR-35360	−	+	−	−
Leptospira fainei -- BUT 6T (Serovar Hurstbridge)	BEI Resources NR-22252	−	+	−	−
Leptospira broomii -- 5399T (Serovar Hurstbridge)	BEI Resources NR-22253	−	+	−	−
Leptospira licerasiae -- VAR10 (Serovar Varillal)	BEI Resources NR-19925	−	+	−	−

Saprophytic					
Leptospira meyeri -- Went 5 (Serovar Hardjo)	BEI Resources NR-29052	−	+	−	−
Leptospira wolbachii -- CDC (Serovar Codice)	BEI Resources NR-35357	−	+	−	−
Other bacteria					
E. coli DH5α	CCB MBL	−	−	−	−
Bacillus sp.	CCB MBL	−	−	−	−
S. aureus	CCB MBL	−	−	−	−

*(ATCC), American Type Culture Collection, (BEI Resources), Biodefense and Emerging Infections Research Resources Repository; (CCB MBL), Centre for Chemical Biology-Microbial Biodiversity Library

Genomic DNA extraction and quality assessment

Briefly, 1.5 mL of the overnight bacterial culture was first centrifuged at 13,000 × *g* for 5 min to obtain approximately 0.1 µg pellet. Their gDNA was then extracted using QIAamp DNA Mini Kit (Qiagen, USA) according to the manufacturer's protocols with minor modifications on the final elution step, in which the column was incubated at room temperature for 10 min prior to centrifugation at 12,000 × *g*. The purity and concentration of the gDNA obtained were determined using the NanoDrop spectrophotometer 2000 (Thermo Scientific, USA) at 260/280 nm absorbance measures and kept at −20 °C for storage.

Primer design

Sequences of the target *Leptospira* genes were downloaded from the Genbank (http://ncbi.nlm.nih.gov/GenbankSearch.html). The sequences were aligned using the Clustal Omega web tool, then species-specific conserved regions for each of the target species were identified, and specific primers were designed based on these regions using Primer-BLAST. The overall theoretical specificities of the newly designed primers were checked using a BLAST search.

The lipL32-917F (5'GCAAGCATTACCGCTTGTGG-3') and lipL32-1162R (5'ACGAACTCCCATTTCAGCGA-3') primers were designed based on conserved regions obtained from multiple sequence alignment of the *lipL32* gene sequence of eight *Leptospira* spp. (Clark *et al.* 2016). The sequences were obtained from the NCBI database, with the following GenBank accession numbers: *Leptospira borgpetersenii* serovar Hardjo (AF181554), *L. interrogans* serovar Autumnalis (AF366366), *L. interrogans* serovar Copenhageni (AF245281), *L. interrogans* serovar Lai (LIU89708), *L. interrogans* serovar Pomona (AF181553), *Leptospira kirschneri* serovar Grippotyphosa (AF121192), *Leptospira*

noguchii serovar Fortbragg (AF181556), and *Leptospira santarosai* serovar Tropica (AF181555). The resulting amplicon was 246 bp between positions 917 and 1162 of the *lipL32* coding regions (Supplementary Figure S1).

The rrs-1011F (5'TCGTGTCGTGAGATGTTGGG-3') and rrs-1009R (5'TGTCACCGGCAGTTCCTTAC-3') primers were designed using multiple sequence alignment of the *rrs* gene sequences of 21 *Leptospira* spp. (Clark *et al.* 2016), conducted with Clustal Omega. The sequences were obtained from the National Centre for Biotechnology (NCBI) database, with the following GenBank accession numbers: *Leptospira alexanderi* serovar Manhao (AY631880, *Leptospira borgpetersenii* serovar Ballum (AM050581), *L. interrogans* serovar Australis (AY996794), *L. interrogans* serovar Autumnalis (AY996791), *L. interrogans* serovar Bataviae (EF536987), *Leptospira kirschneri* serovar Agogo (DQ991476), *Leptospira* mayottensis (KJ847187), DQ991476, *Leptospira kmetyi* serovar Malaysia (AHMP02000003), *Leptospira santarosai* (AY461889), *Leptospira weilii* serovar Celledoni (AY631877), *Leptospira broomii* strain L065 (AY792329), *Leptospira fainei* serovar Hurstbridge (AY996789), *Leptospira inadai* serovar Aguaruna (AY631891), *Leptospira licerasiae* serovar Varillal (EF612278), *Leptospira wolffii* serovar Khorat (EF025496), *Leptospira biflexa* serovar Patoc (AY631876), *Leptospira idonii* (AB721966), *Leptospira meyeri* serovar Hardjo (AY631889), *Leptospira* genomosp. 4 serovar Hualin (AY631888), *Leptospira* genomosp. 3 serovar Holland (AY631897), *Leptospira wolbachii* serovar Codice (AY631879), *Leptospira* genomosp. 5 serovar Saopaulo (AY631882). The resulting amplicon was 99 bp between positions 1011 and 1109 of the *rrs* coding regions (Supplementary Figure S2).

Duplex PCR

The PCR reaction mixture consisted of the HotStarTaq Plus Master Mix kit (Qiagen, USA) with dye, 5 µL DNA template, PCR-grade water, and 200 nM of each forward and reverse primer. The final volume of the PCR reaction mixture was adjusted to 20 µL with PCR-grade water. The final concentration of each primer set (Table 2) was fixed to ensure consistent amplification of each amplicon. Amplification was performed in a thermocycler (Applied Biosystems Veriti, USA) using the following optimized condition: initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1 min, and DNA extension at 72 °C for 1 min before the final extension step at 72 °C for 10 min for complete synthesis of all strands. Then, approximately 2.5 µL of the post-PCR reaction mixture was loaded onto a 2% agarose gel containing RedSafe Nucleic Acid Staining Solution (5 µL/100 mL) (Intron Biotechnology, South Korea). The Thermo Scientific Gene Ruler 100 bp ladder (catalog no. 00998333) was used as the reference for molecular weight. The amplified

products were visualized and photographed under UV light (Bio-Rad Gel Doc, USA). Amplification trends were reproducible across three technical replicates and over three independent testing days, indicating stable assay performance.

Table 2: Details of primers used for *Leptospira* spp. identification.

Primers	Sequences of the primers (5'−3')	Amplicon size (bp)	Ref.
lipL32-917F	GCAAGCATTACCGCTTGTGG	246	This study
lipL32-1162R	ACGAACTCCCATTTCAGCGA		
rrs-1011F	TCGTGTCGTGAGATGTTGGG	99	This study
rrs-1109R	TGTCACCGGCAGTTCCTTAC		
lfb1-F	CATTCATGTTTCGAATCATTTCA	331	Merien *et al.*
lfb1-R	AA		2005
secYIV-F	GGCCCAAGTTCCTTCTAAAAG	202	
secYIV-R	GCGATTCAGTTTAATCCTGC		Ahmed *et al.*
	GAGTTAGAGCTCAAATCTAAG		2009

Spiking experiment

L. interrogans serovar Copenhageni was cultured until reaching an optical density of 0.14 (420 nm) at 30°C. The culture was then centrifuged at room temperature and resuspended in phosphate-buffered saline (PBS). The number of viable leptospires in the suspension was counted using a Petroff-Hausser counting chamber (Fisher Scientific) to achieve a suspension of approximately 5 x 10^8 bacteria/mL. About 400 µL of the suspension was then serially diluted and added to 1.6 mL of a urine sample from a healthy human, creating leptospire-spiked urine samples at final concentrations of 10^0, 10^1, 10^2, 10^3,10^4, 10^5, and 10^6 bacteria/mL and incubated for 2 h at room temperature. Next, 1 mL of each spiked urine sample was concentrated by centrifugation at 13,000 × *g* for 15 min and washed once with 1 mL PBS solution. The gDNA was then extracted from the cells using the QIAamp Mini Kits (Qiagen, USA) based on the above-mentioned modified protocol. The concentration of DNA was determined spectrophotometrically by measurement of the absorbance at 260/280 nm using a Nanodrop 1000 apparatus (Thermo Fisher Scientific). The study was approved by the Human Ethics Committee of Universiti Sains Malaysia (Protocol Code: USM/JEPem/1909055), and all work involving pathogenic *Leptospira* spp. was conducted under BSL-2 containment in a certified facility in accordance with institutional biosafety regulations with IBBC approval.

Sensitivity and detection limit of the primers

The sensitivity and detection limit of the duplex PCR assay consisting of the lipL32-917F/1162R and rrs-1011F/1109R primers were determined using serially diluted *L. interrogans* serovar Copenhageni spiked in urine samples in the spiking experiment and tenfold serially-diluted gDNA suspension of *L. interrogans* serovar Copenhageni in pure water. The serial concentrations were 30 ng/µL, 3 ng/µL, 300 pg/µL, 30 pg/µL, 3 pg/µL, 300 fg/ µL, and 30fg/ µL. For comparison, singleplex PCR was also conducted for the *lfb1* and *secYIV* gene-targeting primers. The identity of each bacterial strain was confirmed using standard biochemical methods and partial 16S rRNA sequencing. Ultrapure water was used as a negative control. The expected PCR amplicon sizes are 246 bp for the *lipL32* gene, 99 bp for the *rrs* gene, 331 bp for the *lfb1* gene, and 202 bp for the *secYIV* gene. DNA concentration was determined spectrophotometrically by measurement of the absorbance at 260/280 nm using a Nanodrop 1000 instrument (Thermo Fisher Scientific).

Specificity of the duplex PCR assay

To evaluate the specificity of the duplex PCR assay consisting of the lipL32-917F/1162R and rrs-1011F/1109R primers, it was tested against nine pathogenic, four intermediately pathogenic, two non-pathogenic *Leptospira*, and three non-leptospiral pathogenic bacteria (Table 1). Singleplex PCR was also conducted using the *lfb1* and *secYIV* gene-targeting primers for comparison. Ultrapure water was included as a negative control, and strain identities were confirmed by biochemical tests and partial 16S rRNA gene sequencing. Sanger sequencing of selected amplicons further validated the specificity of the PCR products.

RESULTS

Sensitivity and Detection limit of the duplex PCR Assay

The rrs-1011F/1109R and lipL32-917F/1162R primers designed enabled the detection of the presence of *Leptospira* and pathogenic *Leptospira*, respectively. The two primer sets were integrated into a duplex PCR setting for simultaneous identification of both categories in a single assay. Comparison of the duplex PCR assay targeting *lipL32* and *rrs* genes with the singleplex PCR assays targeting *lfb1* or *secYIV* genes showed comparable analytical sensitivity. The low detection limit of 10^0 bacteria/mL for urine-spiked *L. interrogans* serovar Copenhageni was recorded for all three assays evaluated in this study (Figure 1A − C). Further evaluation with the extracted gDNA of *L. interrogans* serovar Copenhageni showed successful

detection from 30 fg/uL to 300 ng/uL gDNA (Figure 2A). The detection limit was comparable to 30 fg/μL and 300 fg/μL for the *lfb1* and *secYIV* genes, respectively (Figure 2B − C).

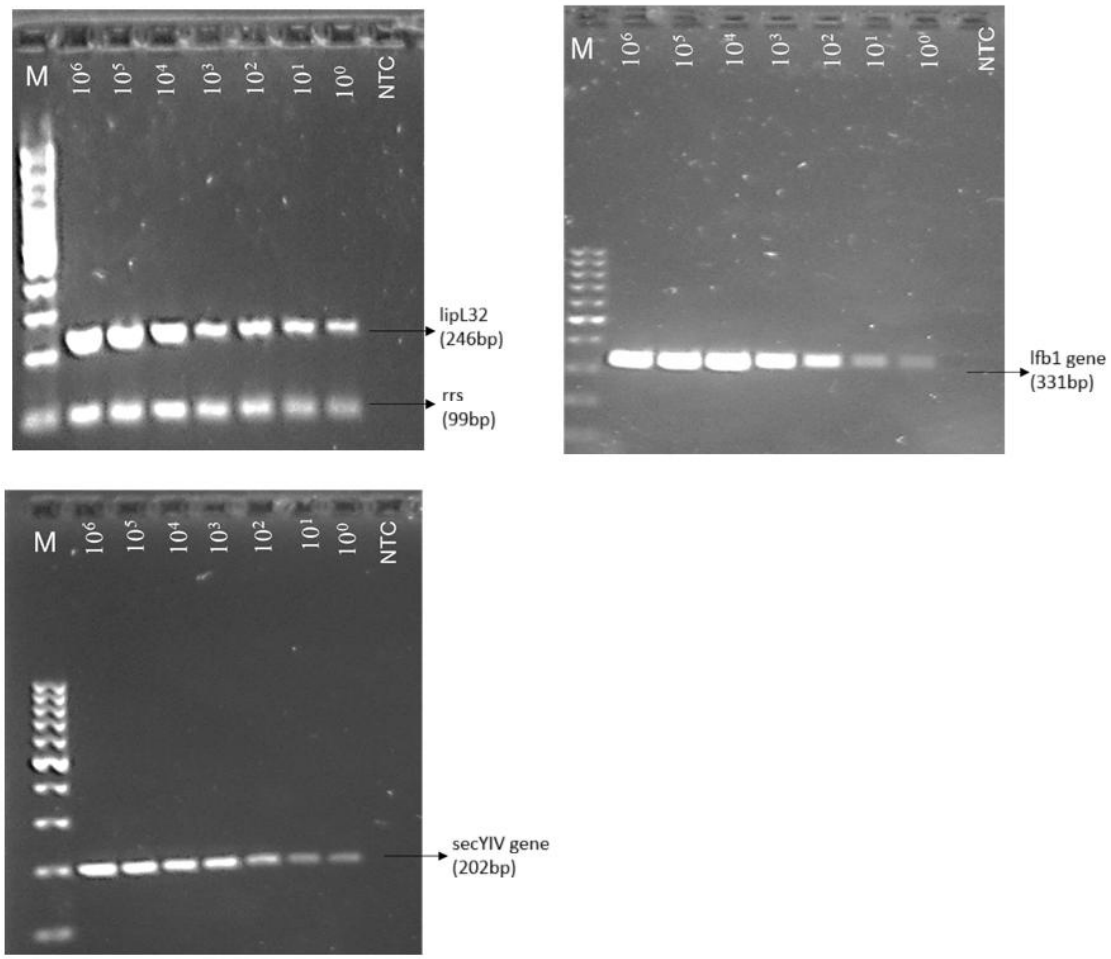

Figure 1: Electrophoresis visualization of the PCR products for sensitivity evaluation of the duplex PCR assay on spiked urine samples, with serial dilution ranging from 10^0 − 10^6 bacteria/ mL. (**A**) The duplex PCR assay targeting *lipl32* and *rrs* genes. (**B**) the singleplex PCR assay targeting *the lfb1* gene, and (**C**) the singleplex PCR assay targeting the *secYIV* gene. **Lane M**: Thermo Scientific Gene Ruler 100 bp ladder.

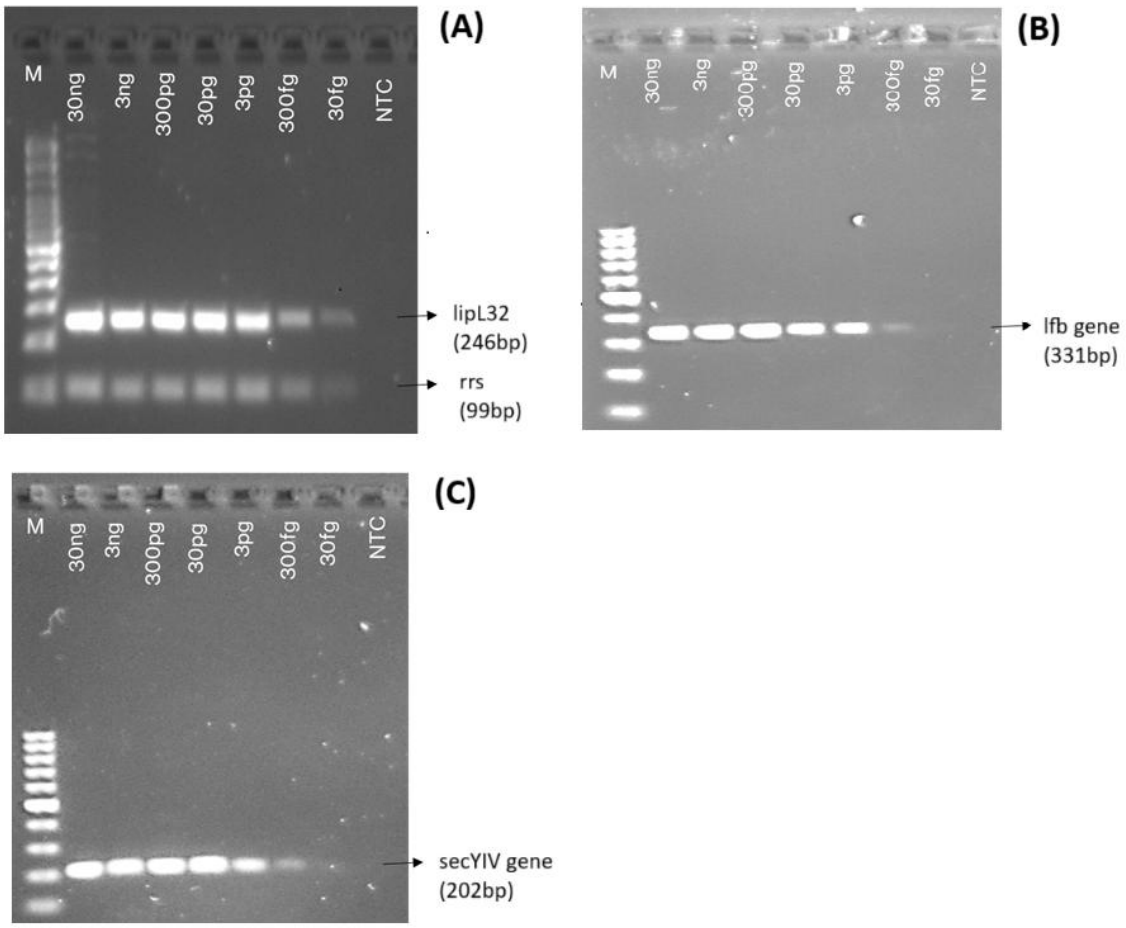

Figure 2: Electrophoresis visualization of the PCR products for sensitivity evaluation of the duplex PCR assay on *L. interrogans* serovar Copenhageni gDNA, with gDNA concentrations of 30 fg/uL – 30 ng/uL. (**A**) The duplex PCR assays targeting the *lipl32* and *rrs* genes, (**B**) the singleplex PCR assay targeting the *lfb1* gene, and (**C**) the singleplex PCR assay targeting the *secYIV* gene. **Lane M**: Thermo Scientific Gene Ruler 100 bp ladder.

Specificity of the duplex PCR assay

Specificity evaluation of the duplex PCR assay was conducted using 15 leptospiral strains, and both the lipL32-917F/1162R and rrs-1011F/1109R primers were 100% specific (Figure 3A, Table 1). Successful detection was observed for all 15 leptospiral strains using the rrs-1011F/1109R primers, while all 9 pathogenic leptospiral strains were identified specifically using the lipL32-917F/1162R primers. Comparatively, the singleplex PCR assay targeting the *lfb1* gene failed to detect *L. noguchii* CZ 214T (Serovar Panama) (specificity = 89%) (Figure 3B, Table 1). Besides, *L. interrogans* -- CSL4002 (Serovar Pomona) and *L. noguchii* CZ 214T (Serovar Panama) were not detected through the singleplex PCR assay targeting the *SecYIV*

gene (specificity = 78%) (Figure 3C, Table 1). No false-positive detection was observed for bacteria other than *Leptospira*. The assay consistently amplified the *rrs* target in all strains and the *lipL32* target exclusively in pathogenic strains, allowing clear differentiation within a single reaction.

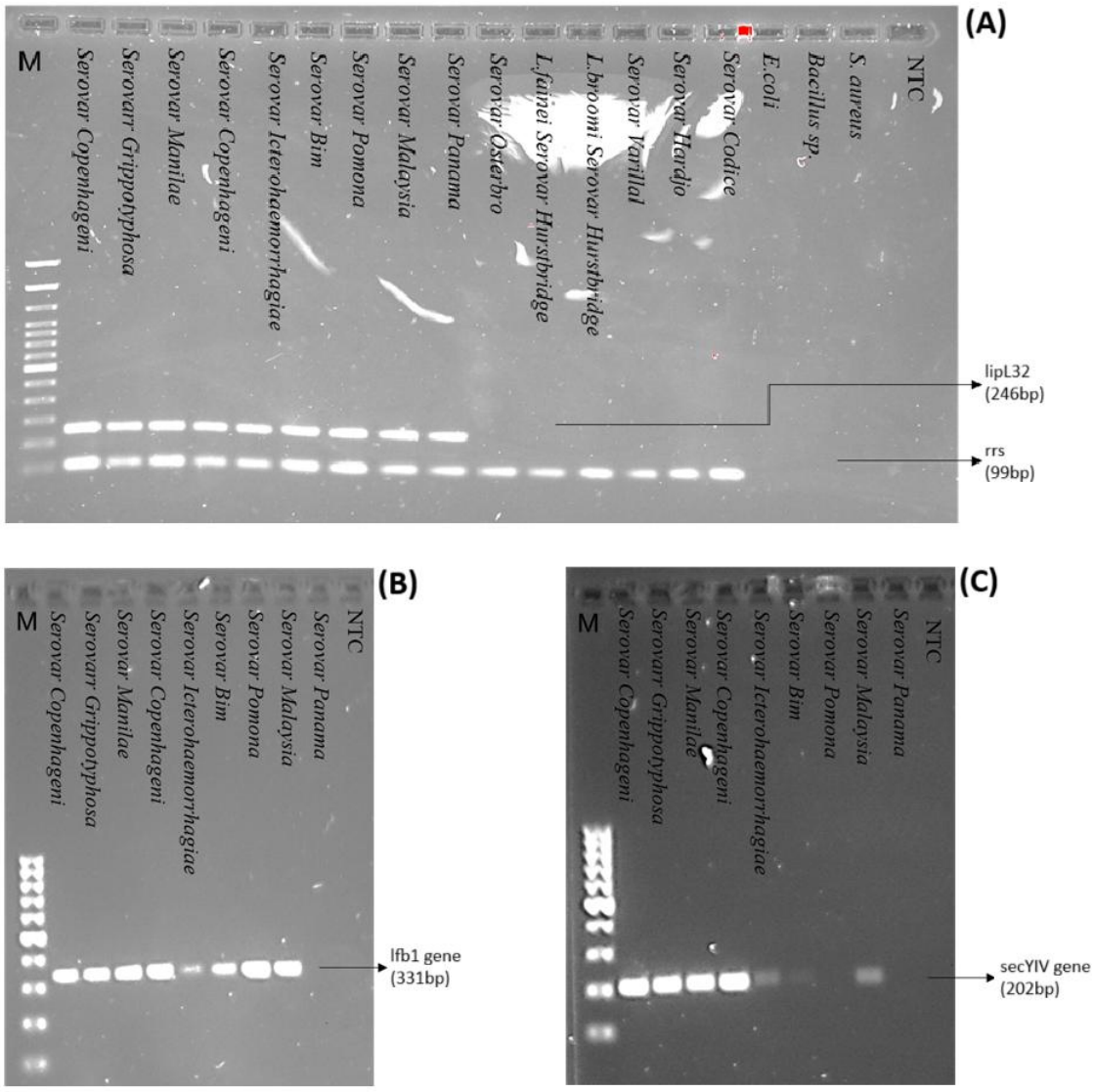

Figure 3. Electrophoresis visualization of the PCR products for the specificity evaluation of the designed primer sets toward 15 *Leptospira* strains. (**A**) The duplex PCR assay targeting *lipl32* and *rrs* genes, (**B**) the singleplex PCR assay targeting the *lfb1* gene, and (**C**) the singleplex PCR assay targeting the *secYIV* gene. **Lane M**: Thermo Scientific Gene Ruler 100 bp ladder, **Lane 1**: *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, **Lane 2**: *L. interrogans* -- 2006006971 (Serovar Grippotyphosa), **Lane 3**: *L. interrogans* -- L495 (Serovar Manilae), **Lane 4**: *L. interrogans* -- HAI0024 (Serovar Icterohaemorrhagiae/Copenhageni), **Lane 5**: *L. interrogans* -- Verdun LP (Serovar Icterohaemorrhagiae), **Lane 6**: *L. kirschneri* --

1051 (Serovar Bim), **Lane 7**: *L. interrogans* -- CSL4002 (Serovar Pomona), **Lane 8**: *L. kmetyi* strain Bejo-Iso9T (serovar Malaysia), **Lane 9**: *L. noguchii* CZ 214T (Serovar Panama), **Lane 10**: *L. hovindhougenii* -- B5-022 (Serovar Osterbro), **Lane 11**: *L. fainei* -- BUT 6T (Serovar Hurstbridge), **Lane 12**: *L. broomii* -- 5399T (Serovar Hurstbridge), **Lane 13**: *L. licerasiae* -- VAR10 (Serovar Varillal), **Lane 14**: *L. meyeri* -- Went 5 (Serovar Hardjo), **Lane 15**: *L. wolbachii* -- CDC (Serovar Codice), **Lane 16**: *E. coli* DH5, **Lane 17**: *Bacillus* sp., **Lane 18**: *S. aureus,* **Lane 19**: non-template control (ntc).

DISCUSSION

Conventional diagnostic methods for leptospirosis, such as MAT and ELISA, provide valuable serological information but have notable limitations. MAT is labour-intensive, requires maintenance of live *Leptospira* cultures, and typically becomes positive only in the later stages of infection when agglutinating antibodies appear (Levett, 2001; Picardeau, 2013). ELISA offers improved accessibility but still depends on host immune responses, which reduces diagnostic sensitivity during the acute phase of infection (Adler & de la Peña Moctezuma, 2010). Molecular assays such as qPCR provide high analytical sensitivity and enable early detection of pathogenic *Leptospira* spp. (Smythe et al., 2002; Ahmed et al., 2009). Yet, their reliance on real-time instrumentation and higher operating costs limits widespread implementation, particularly in resource-limited settings.

The duplex PCR assay developed in this study is a potential alternative in the *Leptospira* diagnosis workflow by offering simultaneous amplification of the *rrs* and *lipL32* genes in a single reaction. This advantage may support earlier pathogen detection using conventional thermocyclers. Although it does not offer quantitative interpretation like qPCR, the duplex assay demonstrates strong specificity while reducing reagent cost and overall workflow time. These attributes give the assay strong translational potential for integration into public health laboratory workflows, including frontline triaging, decentralized diagnostics, and outbreak surveillance in endemic regions (Costa et al., 2015). By providing a rapid and cost-effective molecular tool, the duplex PCR approach may improve the timeliness and accessibility of leptospirosis screening, especially in settings where MAT, ELISA, or qPCR infrastructure is limited.

The successful demonstration of urine-spiked *L. interrogans* serovar Copenhagenii verifies the potential of urine samples for *Leptospira* spp. detection reported previously. As validated in previous studies, the use of human urine samples for the diagnosis of *Leptospira* spp. was demonstrated for real-time PCR, and urine has been suggested as a non-invasive sample (Shukla *et al.* 2021; Villumsen *et al.* 2012). While *Leptospira* spp. are cleared from most tissues after the first day of infection, they can colonize the lumen of the proximal

convoluted tubules of the kidneys within the following weeks (Shukla *et al.* 2021). As *Leptospira* spp. are mostly detectable in the urine from the 7th day of infection, molecular diagnosis of the pathogen from the urine coupled with serological methods could offer enhanced detection efficiency and accuracy (Bourhy *et al.* 2011; Shukla *et al.* 2021). Comparatively, molecular diagnosis of *Leptospira* spp. from urine samples has been verified to show more promising results than serological methods (Othman *et al.* 2019). Successful detection of *Leptospira* spp. was reported in India, where 20% of the samples were diagnosed by targeting the *lipL32* gene (Shukla *et al.* 2021). Moreover, a study conducted in Slovenia reported successful detection of 11.8% of the urine samples, with 6.8% of the diagnoses targeting the *rrs* gene (Podgoršek *et al.* 2020).

The high analytical detection capability of the duplex PCR assay, with low detection limits of 30 fg/uL for gDNA diagnosis and 10^0 bacteria/mL for spiked-urine samples, indicates the potential of the duplex PCR assay as a complementary diagnosis against the well-known diagnostic methods (Figure 3A & 4A). As reported for serum samples, the sensitivities of PCR were 62.0%, 72.7%, and 44.4% for samples collected on days 3 − 8, 9 − 14, and 15 − 42, respectively (de Abreu Fonseca *et al.* 2006). The duplex PCR assay in this study demonstrates sensitive analytical detection in urine samples. Although the sensitivities of MAT, immunoglobulin M enzyme-linked immunosorbent assay (IgM ELISA), and slide agglutination test (SAT) were 69.0 − 79.3% for days 3 – 8, 95.4 – 100% for days 9 – 14, and 77.8 – 88.9% for days 15 − 42, respectively, PCR was reported with the highest specificity of 100% among the four methods (de Abreu Fonseca *et al.* 2006).

Despite the analytical sensitivity and promisingly low detection limit of singleplex PCR targeting *lfb1* or *secYIV* genes, the singleplex PCR assays showed lower specificity of 89% and 78%, respectively (Figure 5B & C). The observed specificity shown for the duplex PCR assay targeting the *lipL32* and *rrs* genes is attributed to the highly-conserved nature of the *lipL32* gene in only pathogenic *Leptospira* spp. and the presence of the *rrs* gene in two copies per *Leptospira* bacterium, which could consequently increase its chance of being amplified (Lam *et al.* 2020; Nascimento *et al.* 2004; Picardeau *et al.* 2008). Proteomic studies have reported that LipL32 is the major surface-exposed outer membrane protein from laboratory-cultured strains and is involved directly in mammalian pathogenesis (Cullen *et al.* 2002; Cullen *et al.* 2004; Cullen *et al.* 2005; Hoke *et al.* 2008). The duplex PCR setting in this study offers simplicity for a quicker, easier-to-perform, less prone to contamination, and analytically sensitive determination of *Leptospira* DNA in clinical samples (Podgoršek *et al.* 2020).

The analytical sensitivity and specificity of the duplex PCR assay were validated through tenfold serial dilutions of *Leptospira* DNA and testing against pathogenic and non-pathogenic *Leptospira* strains and selected non-Leptospira bacteria, respectively. The sole amplification of the intended targets indicates high assay specificity. Although probabilistic

models such as Poisson or binomial distributions are commonly used in digital PCR and single-molecule analyses, such modelling is not typically applied to conventional duplex PCR. Accordingly, assay performance in this study was validated empirically through serial dilution analysis, repeatability assessment, and cross-reactivity screening.

The duplex PCR assay is practically more feasible in terms of speed and cost over advanced molecular platforms such as qPCR and LAMP. Although qPCR remains the benchmark for its high analytical sensitivity and quantitative detection, it requires real-time thermocyclers, fluorescence-based reagents, and well-trained personnel. This limits its implementation in many low-resource or district-level laboratories (Levett 2001; Ahmed et al. 2009; Bourhy et al. 2011). LAMP assays, while rapid and operable under isothermal conditions, require complex multi-primer designs and are prone to non-specific amplification, which can complicate result interpretation and increase reagent and optimization costs (Notomi & Mori, 2015; Kim et al., 2023). In contrast, the duplex PCR assay enables simultaneous detection and differentiation of pathogenic *Leptospira* spp. using standard PCR equipment in a single run. Its analytical performance is decent for early infection screening, allowing faster time-to-result and lower operational costs (Boonsilp et al. 2011; Picardeau 2013). Hence, it is particularly suitable for resource-limited settings for wider diagnostic coverage, improved outbreak responsiveness, and earlier clinical intervention (Costa et al. 2015).

Although the duplex PCR assay demonstrated qualitative sensitivity by gel-based detection, quantitative assessments such as copies/mL determination were not included. As conventional PCR is an endpoint, non-quantitative method, such analyses would require qPCR calibration curves and quantified standards, which were beyond the scope of this development phase. Accordingly, this study focused on defining qualitative detection limits and verifying initial assay performance. Future studies will include quantitative sensitivity measurements, replicate-based precision assessments, and intra- and inter-assay variability analyses to provide a more comprehensive evaluation of assay robustness and diagnostic applicability.

The inferences drawn from this study are limited by the absence of clinical samples from suspected leptospirosis patients. Upon primary evaluation using reference strains and urine-spiked samples, further work will focus on the diagnostic applicability of the duplex PCR assay under real-world circumstances. Future validation will include testing clinical specimens from different infection stages to assess assay robustness, diagnostic sensitivity, and performance in routine public health laboratory workflows.

In conclusion, two novel primer sets were successfully designed for *Leptospira* spp. detection and pathogenicity differentiation. The duplex PCR assay offers a specific and more sensitive detection of pathogenic *Leptospira* spp. compared to singleplex PCR assays

targeting *lfb1* or *secYIV* genes. It enables simultaneous detection of *Leptospira* spp. and pathogenic strain differentiation with 100% sensitivity and specificity. This simple, multiplex, and rapid molecular assay is competitive over conventional detection methods and real-time PCR assays for early leptospirosis diagnosis, allowing improved patient recovery prospects.

ETHICS STATEMENT

The study was approved by the Human Ethics Committee of Universiti Sains Malaysia (Protocol code: USM/JEPem/1909055).

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AUTHORS'; CONTRIBUTIONS

Conceptualization: N.M. and N.-S.L.; Methodology: M.R.M.A., S.A.A., N.O. and A.R.; Software and Resources: E.O.B.B., N.I. and K.G.V.S.; Investigation: N.M. and N.A.M.H.; Writing – Original Draft: N.M. and N.R.; Writing – Review and Editing: N.M. and N.-S.L. All authors have read and approved the final manuscript.

REFERENCES

Adler, B., & de la Peña Moctezuma, A. (2010). Leptospira and leptospirosis. *Veterinary Microbiology*, 140(3–4), 287–296. https://doi.org/10.1016/j.vetmic.2009.03.012

Agampodi, S., Gunarathna, S., Lee, J. S., & Excler, J. L. (2023). Global, regional, and country-level cost of leptospirosis due to loss of productivity in humans. *PLOS Neglected Tropical Diseases,* 17(8), e0011291. https://doi.org/10.1371/journal.pntd.0011291

Ahmed, A., Engelberts, M. F., Boer, K. R., Ahmed, N., & Hartskeerl, R. A. (2009). Development and validation of a real-time PCR for detection of pathogenic Leptospira species in clinical materials. *PLOS ONE*, 4(9), e7093. https://doi.org/10.1371/journal.pone.0007093

Ali, M. R. M., Safee, A. W. M., Ismail, N. H., Sapian, R. A., Hussin, H. M., Ismail, N., & Yean, C. Y. (2018). Development and validation of pan-Leptospira TaqMan qPCR for the detection of Leptospira spp. in clinical specimens. *Molecular and Cellular Probes*, 38, 1–6. https://doi.org/10.1016/j.mcp.2018.03.001

Boonsilp, S., Thaipadungpanit, J., Amornchai, P., Wuthiekanun, V., Chierakul, W., Limmathurotsakul, D., Day, N. P., & Peacock, S. J. (2011). Molecular detection and speciation of pathogenic Leptospira spp. in blood from patients with culture-negative

leptospirosis. *BMC Infectious Diseases*, 11, 338. https://doi.org/10.1186/1471-2334-11-338

Bourhy, P., Bremont, S., Zinini, F., Giry, C., & Picardeau, M. (2011). Comparison of real-time PCR assays for detection of pathogenic Leptospira spp. in blood and identification of variations in target sequences. *Journal of Clinical Microbiology*, 49(6), 2154–2160. https://doi.org/10.1128/JCM.02452-10

Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Sayers, E. W. (2016). GenBank. *Nucleic Acids Research*, 44(D1), D67–D72. https://doi.org/10.1093/nar/gkv1276

Costa, F., Hagan, J. E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M. S., Stein, C., Abela-Ridder, B., & Ko, A. I. (2015). Global morbidity and mortality of leptospirosis: A systematic review. *PLOS Neglected Tropical Dise*ases, 9(9), e0003898. https://doi.org/10.1371/journal.pntd.0003898

Cullen, P. A., Cordwell, S. J., Bulach, D. M., Haake, D. A., & Adler, B. (2002). Global analysis of outer membrane proteins from Leptospira interrogans serovar Lai. *Infection and Immunity*, 70(5), 2311–2318. https://doi.org/10.1128/IAI.70.5.2311-2318.2002

Cullen, P. A., Haake, D. A., & Adler, B. (2004). Outer membrane proteins of pathogenic spirochetes. *FEMS Microbiology Reviews*, 28(3), 291–318. https://doi.org/10.1016/j.femsre.2003.10.004

Cullen, P. A., Xu, X., Matsunaga, J., Sanchez, Y., Ko, A. I., Haake, D. A., & Adler, B. (2005). Surfaceome of Leptospira spp. Infection and Immunity, 73(8), 4853–4863. https://doi.org/10.1128/IAI.73.8.4853-4863.2005de Abreu Fonseca C, Teixeira de Freitas V L, Caló Romero E, Spinosa C, Arroyo Sanches M C, da Silva M V and Shikanai-Yasuda M A. (2006). Polymerase chain reaction in comparison with serological tests for early diagnosis of human leptospirosis. *Tropical Medicine and International Health* 11(11): 1699−1707. https://doi.org/10.1111/j.1365-3156.2006.01727.x

de Abreu Fonseca, C., Teixeira de Freitas, V. L., Caló Romero, E., Spinosa, C., Arroyo Sanches, M. C., da Silva, M. V., & Shikanai-Yasuda, M. A. (2006). Polymerase chain reaction compared with serological tests for early diagnosis of human leptospirosis. *Tropical Medicine & International Health*, 11(11), 1699–1707. https://doi.org/10.1111/j.1365-3156.2006.01727.x

Garcia-Lopez, M., Lorioux, C., Soares, A., Trombert-Paolantoni, S., Harran, E., Ayral, F., Picardeau, M., Djelouadji, Z., & Bourhy, P. (2023). Genetic diversity of Leptospira strains circulating in humans and dogs in France in 2019–2021. *Frontiers in Cellular and Infection Microbiology*, 13, 1236866. https://doi.org/10.3389/fcimb.2023.1236866

Goris, M. G., & Hartskeerl, R. A. (2014). Leptospirosis serodiagnosis by the microscopic agglutination test. *Current Protocols in Microbiology*, 32(1), 12E.5. https://doi.org/10.1002/9780471729259.mc12e05s32

Haake, D. A., & Levett, P. N. (2015). Leptospirosis in humans. In B. Adler (Ed.), Leptospira and leptospirosis (pp. 65–97). *Springe*r. https://doi.org/10.1007/978-3-662-45059-8_5

Hagedoorn, N. N., Maze, M. J., Carugati, M., Cash-Goldwasser, S., Allan, K. J., Chen, K., Galloway, R. L. (2024). Global distribution of Leptospira serovar isolations and detections from animal hosts: A systematic review and online database. *Tropical Medicine & International Health*, 29(3), 161–172. https://doi.org/10.1111/tmi.13965

Hoke, D. E., Egan, S., Cullen, P. A., & Adler, B. (2008). LipL32 is an extracellular matrix-interacting protein of Leptospira spp. and Pseudoalteromonas tunicata. *Infection and Immunity*, 76(5), 2063–2079. https://doi.org/10.1128/IAI.01643-07

Jaiswal, N. K., Chandrasekaran, S., & Rukadikar, A. (2019). Usefulness of dark-field microscopy, IgM ELISA and microscopic agglutination test for early diagnosis of acute leptospirosis. *Indian Journal of Microbiology Research*, 6, 166–169. https://doi.org/10.18231/j.ijmr.2019.036

Kim, S. H., Lee, S. Y., Kim, U., & Oh, S. W. (2023). Diverse methods of reducing and confirming false-positive results of loop-mediated isothermal amplification assays: A review. *Analytica Chimica Acta*, 1280, 341693. https://doi.org/10.1016/j.aca.2023.341693

Lam, J. Y., Low, G. K. K., & Chee, H. Y. (2020). Diagnostic accuracy of genetic markers and nucleic acid techniques for detection of Leptospira in clinical samples: A meta-analysis. *PLOS Neglected Tropical Diseases*, 14(2), e0008074. https://doi.org/10.1371/journal.pntd.0008074

Landolt, N. Y., Chiani, Y. T., Pujato, N., Jacob, P., Schmeling, M. F., Effron, G. G., & Vanasco, N. B. (2023). Utility evaluation of two molecular methods for Leptospira typing in human serum samples. *Heliyon*, 9(2), e12564. https://doi.org/10.1016/j.heliyon.2022.e12564

Levett, P. N. (2001). Leptospirosis. *Clinical Microbiology Reviews*, 14(2), 296–326. https://doi.org/10.1128/CMR.14.2.296

Merien, F., Portnoi, D., Bourhy, P., Charavay, F., Berlioz-Arthaud, A., & Baranton, G. (2005). A rapid and quantitative method for detection of Leptospira species in human leptospirosis. *FEMS Microbiology Letters*, 249(1), 139–147. https://doi.org/10.1016/j.femsle.2005.06.011

Mori, Y., & Notomi, T. (2015). Loop-mediated isothermal amplification (LAMP): Principle, features, and future prospects. *Journal of Microbiology*, 53(1), 1–5. https://doi.org/10.1007/s12275-015-4656-9

Nascimento, A. L. T. O., Verjovski-Almeida, S., Van Sluys, M. A., Monteiro-Vitorello, C. B., Camargo, L. E. A., Digiampietri, L. A., … Setubal, J. C. (2004). Genome features of Leptospira interrogans serovar Copenhageni. *Brazilian Journal of Medical and Biological Research,* 37, 459–477. https://doi.org/10.1590/S0100-879X2004000400003

Natarajan, S., Joseph, J., Vinayagamurthy, B., & Estrela, P. (2023). A lateral flow assay for detection of Leptospira lipL32 gene using CRISPR technology. *Sensors*, 23(14), 6544. https://doi.org/10.3390/s23146544

Othman, S., Philip, N., Taib, N. M., Neela, V. K., & Chee, H. Y. (2019). Detection of leptospiral DNA in urine sample following prolonged hospitalization: A case report. *Malaysian Journal of Medicine and Health Sciences*, 15(SP2), 105–107. http://psasir.upm.edu.my/id/eprint/70062.

Pérez, L. J., Lanka, S., DeShambo, V. J., Fredrickson, R. L., & Maddox, C. W. (2020). A validated multiplex real-time PCR assay for diagnosis of infectious Leptospira spp. *Frontiers in Microbiology*, 11, 457. https://doi.org/10.3389/fmicb.2020.00457

Picardeau, M. (2013). Diagnosis and epidemiology of leptospirosis. *Médecine et Maladies Infectieuses*, 43(1), 1–9. https://doi.org/10.1016/j.medmal.2012.11.005

Picardeau, M., Bulach, D. M., Bouchier, C., Zuerner, R. L., Zidane, N., Wilson, P. J., McGrath, A. (2008). Genome sequence of the saprophyte Leptospira biflexa provides insights into the evolution of Leptospira and pathogenesis of leptospirosis. *PLOS ONE*, 3(2), e1607. https://doi.org/10.1371/journal.pone.0001607

Piredda, I., Bertoldi, L., Benvenuto, G., Palmas, B., Pedditzi, A., Pintore, P., & Chisu, V. (2021). First isolation and molecular typing of pathogenic and intermediate Leptospira species from urine of symptomatic dogs. *Veterinary Sciences*, 8(12), 304. https://doi.org/10.3390/vetsci8120304

Podgoršek, D., Ružić-Sabljić, E., Logar, M., Kobal, S., Retelj, M., & Avšič-Županc, T. (2020). Evaluation of real-time PCR targeting the lipL32 gene for diagnosis of Leptospira infection. *BMC Microbiology*, 20, 59. https://doi.org/10.1186/s12866-020-01744-4

Puche, R., Ferrés, I., Caraballo, L., Rangel, Y., Picardeau, M., Takiff, H., & Iraola, G. (2018). Leptospira venezuelensis sp. nov., a new member of the intermediate group isolated from rodents, cattle and humans. *International Journal of Systematic and Evolutionary Microbiology,* 68(2), 513–517. https://doi.org/10.1099/ijsem.0.002528

Rajapakse, S., Fernando, N., Dreyfus, A., et al. (2025). Leptospirosis. *Nature Reviews Disease Primers*, 11, 32. https://doi.org/10.1038/s41572-025-00614-5

Shukla, S., Mittal, V., Singh, P., & Singh, A. (2021). Evaluation of TaqMan-based real-time PCR assay targeting lipL32 gene for leptospirosis in serologically positive human urine samples from north India. *Indian Journal of Medical Microbiology*, 39(1), 11–14. https://doi.org/10.1016/j.ijmmb.2020.10.017

Smythe, L. D., Smith, I. L., Smith, G. A., Dohnt, M. F., Symonds, M. L., Barnett, L. J., & McKay, D. B. (2002). A quantitative PCR (TaqMan) assay for pathogenic Leptospira spp. *BMC Infectious Diseases*, 2, 13. https://doi.org/10.1186/1471-2334-2-13

Smythe, L. D., Wuthiekanun, V., Chierakul, W., Suputtamongkol, Y., Tiengrim, S., Dohnt, M. F., … Day, N. P. (2009). The microscopic agglutination test is an unreliable predictor of infecting Leptospira serovar in Thailand. *The American Journal of Tropical Medicine and Hygiene*, 81(4), 695–697. https://doi.org/10.4269/ajtmh.2009.09-0252

Soo, Z. M. P., Khan, N. A., & Siddiqui, R. (2020). Leptospirosis: Increasing importance in developing countries. *Acta Tropica*, 201, 105183. https://doi.org/10.1016/j.actatropica.2019.105183

Villumsen, S., Pedersen, R., Borre, M. B., Ahrens, P., Jensen, J. S., & Krogfelt, K. A. (2012). Novel TaqMan PCR for detection of Leptospira species in urine and blood: Pitfalls of in silico validation. *Journal of Microbiological Methods*, 91(1), 184–190. https://doi.org/10.1016/j.mimet.2012.06.009

Vincent, A. T., Schiettekatte, O., Goarant, C., Neela, V. K., Bernet, E., Thibeaux, R., … Nakao, R. (2019). Revisiting the taxonomy and evolution of pathogenicity of the genus Leptospira through the prism of genomics. *PLOS Neglected Tropical Diseases,* 13(5), e0007270. https://doi.org/10.1371/journal.pntd.0007270

Vinetz, J. M. (2001). Leptospirosis. *Current Opinion in Infectious Diseases*, 14, 527–538. https://doi.org/10.1097/00001432-200110000-00005

Yeoh, T. S., Tang, T. H., & Citartan, M. (2023). Isolation of a novel DNA aptamer against LipL32 as a potential diagnostic agent for detection of pathogenic Leptospira. *Biotechnology Journal*, 18(3), 2200418. https://doi.org/10.1002/biot.202200418

World Health Organization. (2010). Report of the first meeting of the Leptospirosis Burden Epidemiology Reference Group (LERG). WHO Press.

World Health Organization. (2020). Ending the neglect to attain the Sustainable Development Goals: A road map for neglected tropical diseases 2021–2030. WHO Press.

SUPPLEMENTARY MATERIALS

Figure S1. Locations of forward primer (yellow) and reverse primer (green) for lipL32 gene amplification.

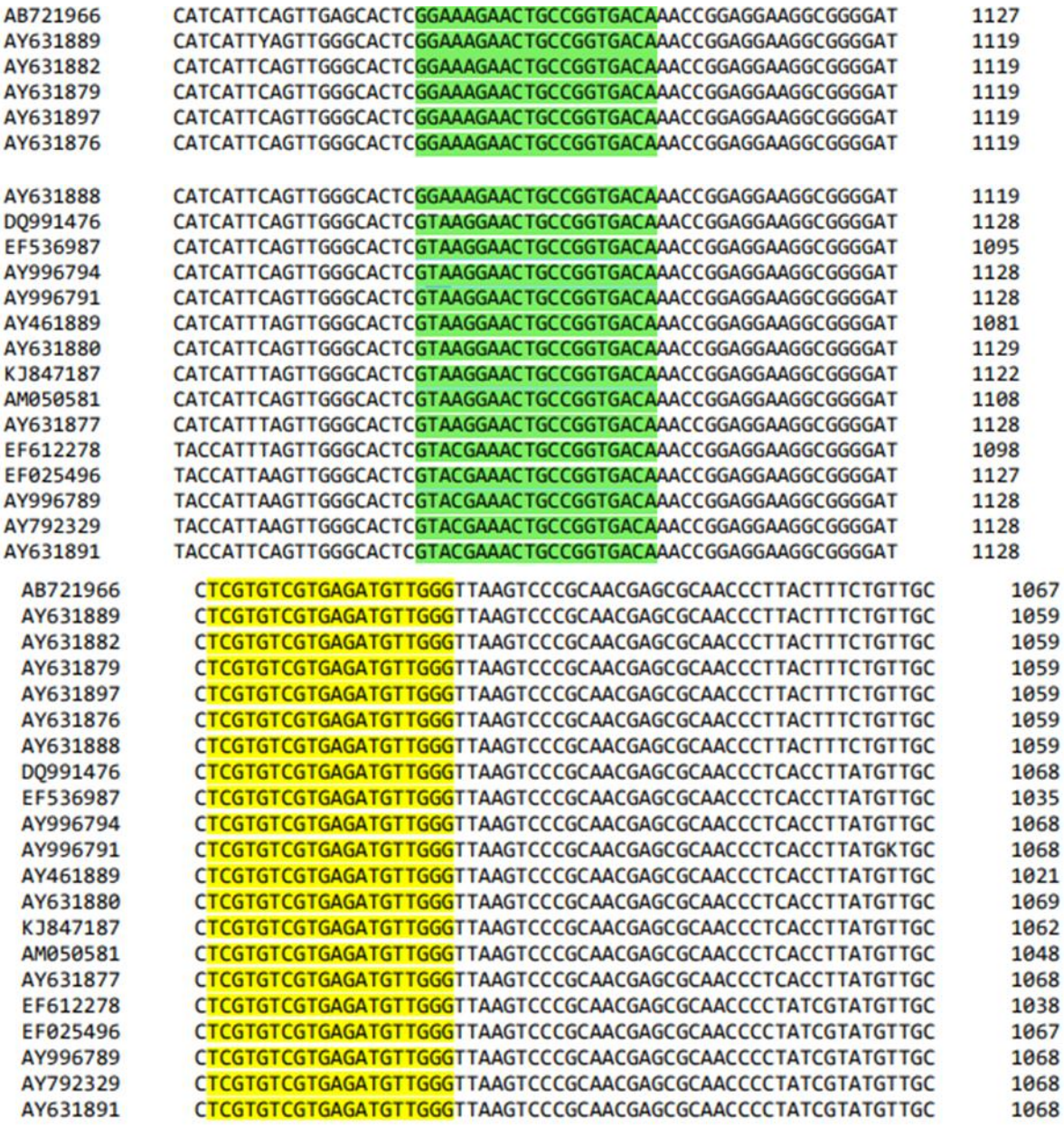

Figure S2. Locations of forward primer (yellow) and reverse primer (green) for rrs gene amplification.