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Quantitative Real-Time Pcr Assays For The Detection Of Leptospira

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Quantitative Real-Time PCR Assays for the Detection of *Leptospira*

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Yale School of Public Health, Department of Epidemiology of Microbial Diseases

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ABSTRACT

Leptospirosis is an infectious zoonotic disease caused by bacteria of the genus *Leptospira*. The genus is comprised of 40 pathogenic species divided among P1 (19) and P2 (21). Leptospirosis incurs a disproportionately large burden of disease in urban slum regions of developing countries that lack adequate sewage disposal, water treatment, and sanitation infrastructure. While P1 species have been identified in cases of human infection, it is unclear if all *Leptospira* species are capable of infecting humans, especially P2 species. Among P1 species, *L. interrogans* has been described as the most frequent specie to cause disease in both animals and humans, and varying pathogenicity has been identified in other pathogenic species. Real-time qPCR assays have been shown to identify *Leptospira* with high sensitivity and specificity; however, there is a lack of assays capable of targeting relevant species of *Leptospira* of specific clinical and environmental importance among both humans and animals. Thus, the purpose of this study was three-fold: (1) design a multiplex assay for the simultaneous detection of and discrimination between P1 and P2 species; (2) optimize a previous assay for the detection of all currently-named P1 species; and (3) create an assay for the specific detection of *L. interrogans*. Primers and probes were designed *in silico* utilizing the BioEdit software to evaluate sequences of all 68 species with available genomic sequences. *In vitro* sensitivity and specificity analyses were conducted on 28 reference species. Given the specificity and sensitivity of the developed assays, real-time qPCR assays may be useful in future epidemiological and clinical studies for the identification and quantification of various *Leptospira* species.

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INTRODUCTION

Leptospirosis is the most widespread zoonosis globally, with over 1 million cases annually[1]. The under-recognition of leptospirosis as a serious public health issue has contributed to its high morbidity and mortality, especially in resource-poor countries in tropical regions. The causative agents of leptospirosis are spirochetes of the genus *Leptospira*, which are transmitted through the urine of either zoonotic reservoirs, such as rodents, sylvatic animals, domestic animals, and livestock, or infected humans. Water and soil contaminated with the urine of an infected animal may penetrate abraded skin or mucous membranes of humans, leading to infection[2]. Leptospirosis infection appears clinically as a nonspecific, acute illness with symptoms such as fever and headache. Infection may ultimately result in widespread hematogenous dissemination of *Leptospira* and/or renal dysfunction[3]. Varying species of *Leptospira* may act as asymptomatic carriers or cause disease with different levels of severity[4]. There are currently 68 named species of *Leptospira*[5].

Advances in whole-genome sequencing have allowed for the recent phylogenetic categorization of the genus *Leptospira* into the following distinct lineages: group I pathogenic (P1), group II pathogenic (P2), group I saprophytic (S1), and group II saprophytic (S2). The saprophytic groups, S1 and S2, are composed of species that thrive in soil and/or water and are not responsible for infections in humans or animals. The pathogenic groups, P1 and P2, are composed of species that may causeinfections in animals and/or humans, as well as environmental species with unknown virulence. Among P1 species, *L. interrogans* has been described as the most frequent specie to cause diseasein both humans and animals[6]. Conversely, although some P2 species have been associated with mild to severe leptospirosis among humans[7-10], the cluster is not well characterized. Thus, many aspects of the epidemiology and virulence of P2 species remain

unknown.

Few quantitative real-time polymerase chain reaction (qPCR) assays have been able to specifically detect pathogenic *Leptospira* species. The *lipl32* gene of pathogenic *Leptospira* species, which encodes an outer membrane lipoprotein, has been described as a putative virulence factor absent in saprophytic species[11,12]. This specificity to pathogenic species distinguished the *lipL32* gene as a suitable and commonly-used target in previous assays designed to identify *Leptospira* species of P1 clades[13,14]. *23S* ribosomal RNA molecules, which display considerable conservation in P1 and P2 species but sequence divergence in saprophytic species, present as a promising target for PCR-based detection of and distinguishment between P1 and P2 species[15]. However, given that most diagnoses are based on serology assays, especially the microscopic agglutination test (MAT) or immunoglobin M enzyme-linked immunosorbent assay (IgM ELISA)[16,17], there is a lack of qPCR assays capable of detecting *Leptospira* species from the P2 clade or identifying serovars among *L. interrogans* specifically. Therefore, this presents a critical need for the development of robust detection tools capable of identifying *Leptospira* species in various contexts, as well as the improvement of previously designed qPCR assays to account for more recently discovered species.

The aim of this study was to develop three rapid and simple assays for the real-time detection of *Leptospira* species with applicability in various laboratory contexts and epidemiological studies. The first assay is a multiplex qPCR design that targets *23S* rRNA and utilizes two distinct probe sequences with different reporters to distinguish between the presence of P1 and P2 species. The second assay, which targets the *lipL32* gene, identifies samples containing all currently named pathogenic *Leptospira* species of the P1 clade. The third and final assay utilizes primers and a probe with high specificity to identify infection caused by different serovars among the *L.*

interrogans specie.

MATERIALS AND METHODS

Bacterial cultures

Strains of *Leptospira spp*. were cultivated in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium and kept up to seven days at 30°C in agitation at 100 rpm[18,19]. Leptospires were inspected weekly under dark-field microscopy and checked for contamination. Samples containing 5 mL of leptospires in late-log phase were centrifuged at 12,000 rpm for 10 minutes. The cells were washed twice with phosphate-buffered saline (PBS) and pelleted at 12,000 rpm for 10 minutes.

DNA extraction methods

Individual pellets of *Leptospira* spp. cultures were frozen at -80°C until extraction. DNA was extracted using the QIAmp DNA minikit (QIAGEN, Valencia, CA) and then quantified with a NanoDrop instrument (Thermo Fisher Scientific Inc., Waltham, MA.). Each DNA sample was diluted such that each sample contained 10^6 copies of equivalent genomic DNA (GEq) per 5 µL of extract based on the size of each genome. Genome sizes were retrieved from the National Center for BiotechnologyInformation (NCBI) Genome webpage.

Alignment of sequences and in silico analysis

Genome sequences were downloaded from the NCBI website and uploaded into the Galaxy software[20]. Sequences of *23S, lipL32,* and *ompL37* genes of *L. interrogans* were downloaded from the Microbial Genome Annotation and Analysis Platform and uploaded into the Galaxy software[21]. The “NCBI BLAST + blastn” function of the Galaxy software was used to locate the sequence of each gene for each respective species. The sequences for each strain were aligned

using the BioEdit software[22]. Following primer and probe design, to avoid primer-dimer or probe-dimer formation, all primer and probe sequences were analyzed for potential interactions and/or secondary structures using NET Primer analysis softwareby PREMIER Biosoft[23]. *In silico* evaluation was performed to ensure reaction efficiency, and all primer and probe sequences were evaluated using BLASTn search of the NCBI database to confirm specificity to *Leptospira* species.

Development of the *23S* assay

The goal of the *23S* rRNA-based assay was to develop and validate a multiplex design to simultaneously detect and distinguish between P1 and P2 (but not saprophytic) *Leptospira* species. The *23S* rRNA sequence was selected due to a previous analysis that identified the gene target as a region that could successfully discriminate between pathogenic and saprophytic *Leptospira* species[15]. A target sequence for the development of probes specific to P1 and P2 species was selected with high variability between the P1 and P2 groups but high conservation within each group. Regions flanking this target sequence were analyzed for primer optimization, with high conservation between P1 and P2 species but sequence divergence from saprophytic species. We designed a single pair of primers able to amplify sequences from both P1 and P2, while the probes were designed for individual identification of P1 and P2, and conjugated with FAM and JOE fluorophore, respectively.

Optimization of the *lipL32* assay

The primers and probes designed targeting the *lipL32* gene aimed to identify samples containing all 19 P1 species. The *lipL32* gene is absent in S1 and S2 species but is present in all P1 and P2 species. We performed analysis using Primer Express™ Software v3.0.1 to optimize a previous primer set created by Thibeaux et al., which is an adaptation of a 2009 design by Stoddard et

al[24,25]. The forward primer of the previous design was shifted by four nucleotides to avoid mismatches with more recently discovered P1 species. Furthermore, position 54 was changed from a C nucleotide to a degenerate nucleotide, M, which is able to bind to both A and C. This alteration was performed due to the presence of an A nucleotide in the 54 position of *L. kmetyi* and *L. stimsonii*, two recently discovered species. The probe and reverse primer designed by Thibeaux et al. were used in combination with this revised forward primer. The probe was kept as the same previously published by Stoddard *et al*.

Development of the *ompL37* assay

The *ompL37* gene was selected as a target for a qPCR assay that would detect only *L. interrogans* and no other species due to its ability to highly discriminate this species of leptospires among others. Primers and probe were designed to bind to the *ompL37* gene of *L. interrogans* with a maximum number of discrepancies with the sequences of all other species, including other P1 species. The designed primers and probe, conjugated with FAM fluorophore, were then aligned with the *ompL37* gene of various *L. interrogans* to ensure that they would detect all serovars of *L. interrogans.*

PCR assay

PCR assays were performed with melting temperature gradients to approximate melting temperatures that optimized primer amplification efficiency. To prepare the PCR reaction mix for each sample, 2.5 μL of 10x PCR NEB Standard Taq buffer was added in addition to 0.2 nM dNTPs (NEB dNTP Solution Mix N0447S), 0.2 μM of appropriate forward primer, 0.2 μM of appropriate reverse primer, and 0.25 μL of NEB Taq Polymerase (M0723L). PCR amplification products resolved onto ethidium bromide mixedagarose gel, electrophoresed in 1X TBE buffer and imaged under UV light in the gel doc XR+ system (Biorad).

qPCR assay (*23S*)

When assessing the efficacy of the multiplex assay targeting *23S* rRNA, the sensitivity of each respective probe was analyzed individually and compared to the multiplex assay. After testing various primer and probe concentrations, the lowest concentrations that still retained for high reaction efficiency were selected. All singleplex reaction mixtures composed of 12.5 μL of Platinum qPCR SuperMix (LifeTechnologies), 500 nM of forward and reverse primer, 100nM of probe, 5 μL of DNA, and q.s.p. of ultrapure water for a total volume of 25 μL. For samples simultaneously assessing detection of two species, 2.5 μL of DNA for each respective species were added. All multiplex reaction mixtures composed of 12.5 μL of Platinum qPCR SuperMix (LifeTechnologies), 500 nM of forward and reverse primer, 100 nM of each respective probe, 5 μL of DNA, and q.s.p of ultrapure water for a total volume of 25 μL (Table 1). The assays were subjected to conditions of 2 min at 50°C, 2 min at 95°C, and 50 total cycles of amplification (15 sec at 95°C and 1 min at 52°C). Each row of each assay included a single non-template control, all of which displayed undetermined detection target sequences. Samples with a cycle threshold (Ct) value below 40 were interpreted as positive.

Table 1. Composition of *23S* qPCR multiplex assay reaction mix for the detection of and differentiation between P1 and P2 species. The multiplex assay reaction mix contained separate probes (23S_P1 and 23S_P2) to individually identify P1 and P2 species simultaneously, while the singleplex assay reaction mix contained either 23S_P1 probe or 23S_P2 probe to identify P1 or P2 species.

	Reagent name	Volume (uL)	Final Concentration
Multiplex	Invitrogen SuperMix w/o ROX	12.5	-
	23S_F Primer	1.25	500 nM
	23S_R Primer	1.25	500 nM
	23S_P1 Probe (FAM)	0.5	100 nM
	23S_P2 Probe (JOE)	0.5	100 nM
	Ultrapure water	4.0	-
	Total	20	-
Singleplex	Invitrogen SuperMix w/o ROX	12.5	-
	23S_F Primer	1.25	500 nM
	23S_R Primer	1.25	500 nM
	23S_P1 Probe (FAM) OR 23S_P2 Probe (JOE)	0.5	100 nM
	Ultrapure water	4.0	-
	Total	20	-

qPCR assay (*lipL32* and *ompL37*)

All assays utilized reaction mixtures that composed of 12.5 μL of Platinum qPCR SuperMix (Life Technologies), 500 nM of forward and reverse primer, 100 nM of probe, 5 μL of DNA, and q.s.p. of ultrapure water for a total volume of 25 μL. Each qPCR was performed using a 7500 Fast Real-Time PCR System (Life Technologies). The assays were subjected to conditions of 2 min at 50°C, 2 min at 95°C, and 40 total cycles of amplification (15 sec at 95°C and 1 min at 60°C). Each row of each assay included a single non-template control, all of which displayed undetermined detection target sequences. Samples with a Ct value below 40 were interpreted as positive.

Limit of Quantification and Limit of Detection

We defined the limit of detection as the lowest sample concentration that can be distinguished from a negative control by the reaction assay at 95% confidence. A serial three-fold dilution was performed, ranging from 10^7 copies/μL to 1 copy/μL. Further, we defined the limit of quantification as the lowest sample concentration that can be reliably quantified by the reaction assay at 95% confidence. A serial three-fold dilution was performed, ranging from 10^7 copies/μL to 1 copy/ μL. Linear regression models, including the coefficient of determination, were fit to examine copy number log(10) of each assay verses the average Ct value across the serial dilution. All statistical analyses were performed using R4.0.2.

RESULTS

qPCR design and *in silico* evaluation

Each target gene was analyzed for appropriate regions for probe and primer design (Figure 1). In order to identify an appropriate region for probe design in the assay targeting the *23S* gene, we sought a target region of the *23S* sequence with high discrimination between P1, P2, and saprophytic species optimal for group-specific probe design (Table 2). The region of the P1-specific probe, **23S_P1**¸ had an average of 10.944 mismatches with P2 species and 12.963 mismatches with saprophytic species, while maintaining 100% homology among all P1 species. The P2-specific probe, **23S_P2**, had an average of 10.167 mismatches with P1 species and 9.074 mismatches with saprophytic species, while maintaining considerable homology among P2 species. Among P2 species, *L. perolatii, L. fluminis,* and *L. fletcheri* had 2 mismatched nucleotides with the P2 probe sequence, while *L. wolffii, L. broomii, L. inadai,* and *L. fainei* each had one mismatched nucleotide with the P2 probe sequence. Regions flanking the target probe region were screened for primer design with high homology between P1 and P2 species and low

shared identity with all saprophytic species. The forward primer design, **23S_F**, targeted a sequence of 100% identity between P1 and P2 species, as well as an average of 2.852 mismatches with saprophytic species. Similarly, the reverse primer design, **23S_R**, targeted another region of 100% identity between P1 and P2 species and an average of 2.815 mismatches with saprophytic species.

Table 2. Primer and probe sequences designed and/or optimized for qPCR-based detection of certain *Leptospira* species. In accordance with International Union of Pure and Applied Chemistry (IUPAC) nucleotide codes, W encodes bases A or T, R encodes bases A or G, and M encodes bases A or C.

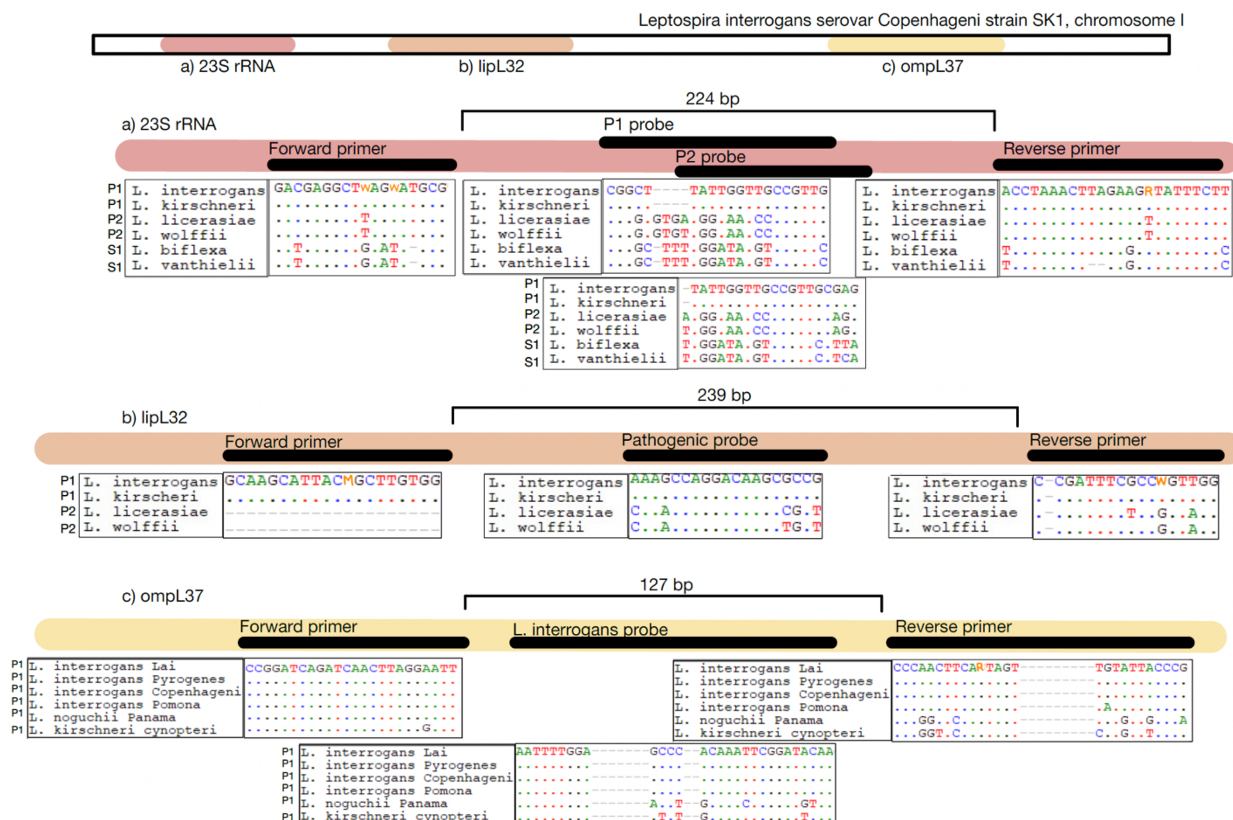
Gene Target	Primer/Probe	Sequence (5' → 3')	*Leptospira* Target
23S	23S_F	GAC-GAG-GCT-WAG-WAT-GCG	*Leptospira* Pathogenic Group I and/or Group II
	23S_R	ACC-TAA-ACT-TAG-AAG-RTA-TTT-CTT	
	23S_P1	^{FAM}CGG-CTT-ATT-GGT-TGC-CGT-TG	*Leptospira* Pathogenic Group I
	23S_P2	^{JOE}ATG-GTA-ATC-CCC-GTT-GCA-GG	*Leptospira* Pathogenic Group II
lipL32	lipL32_F	GCA-AGC-ATT-ACM-GCT-TGT-GG	*Leptospira* Pathogenic Group I
	lipL32_R	CCG-ATT-TCG-CCW-GTT-GG	
	lipL32_P1	^{FAM}AAA-AGC-AGG-ACA-AGC-GCC-G	*Leptospira* Pathogenic Group I
ompL37	ompL37_F	CCG-GAT-CAG-ATC-AAC-TTA-GGA-ATT	*Leptospira interrogans*
	ompL37_R	CCC-AAC-TTC-ART-AGT-TGT-ATT-ACC-CG	
	ompL37_Int	^{FAM}AAT-TTT-GGA-GCC-CAC-AAA-TTC-GGA-TAC-AA	*Leptospira interrogans*

The *lipL32* gene was selected as an appropriate gene target for the detection of P1 species because of its utility in previously designed assays[14,24]. In order to account for recently discovered P1 species since the update of the assay in 2018, the previous design was optimized by shifting the forward primer by four nucleotides (**lipL32_F**) while utilizing an identical reverse primer (**lipL32_R**) and P1 probe (**lipL32_P1**) design. Following these optimization strategies, both the primer and the probe sequences were absent of any mismatches among P1 species. Because P2, S1, and S2 species all lacked the regions targeted by both the forward primer and the P1 probe

utilized by the *lipL32* assay, the assay design suggested high specificity for only P1 species.

In order to confirm the specificity of the *ompL37* assay for only *L. interrogans*, an alignment of all available *L. interrogans* strains were generated alongside the *ompL37* gene sequences of other species. Given that *ompL37* displays low conservation between various species, including those within the P1 clade, it presented as an optimum target for discrimination between *L. interrogans* and other closely related P1 species such as *L. kirschneri* and *L. noguchii*. Further, given that *L. kirschneri* is the closest phylogenetic relative of *L. interrogans*, *L. kirschneri* displayed the highest homology with *L. interrogans* in *ompL37* target regions for primer and probe design. Despite having the lowest number of mismatches with the primer and probe sequences targeting *L. interrogans*, *L. kirschneri* had 7 mismatches with the reverse primer, **ompL37_R**, and 4 mismatches with the *L. interrogans*- specific probe, **ompL37_Int**.

Figure 1. Sequence alignment for each primer/probe pair using representative species. The sequences of relevant representative species from each clade were aligned to assess for mismatches between primers and probes with specie sequences. Aligned nucleotides are represented using a dot, while mismatches are represented by nucleotide codes. Degenerate nucleotide sequences are displayed in orange.



Specificity Analysis

The target specificity of the *23S*-based assay was two-fold: proper amplification of P1 species but no amplification of P2 species or saprophytic species using **23S_P1** probe, as well as proper amplification of P2 species but no amplification of P1 species or saprophytic species using **23S_P2**

probe. Two representative species from each clade were included and tested in triplicates: *L. interrogans* and *L. kirschneri* (P1), *L. licerasiae* and *L. wolffii* (P2), *L. biflexa* and *L. vanthielli* (S1). Various melting temperatures were utilized to determine the one with highest degree of specificity for selected targets without compromising the efficiency of the reaction. Overall, a melting temperature of 52°C resulted in an average Ct of 21.66 for 10^6 copies/μL of *L. interrogans* detected by the **23S_P1** probe and average Ct of 19.43 of 10^6 copies/μL of *L. kirschneri* detected by the **23S_P1** probe (Table 3). *L. licerasiae, L. wolffii, L. biflexa,* and *L. vanthielli*, each at a concentration of 10^6 copies, resulted in an undetermined signal from the **23S_P1** probe. The same assay resulted in an average Ct of 20.17 for 10^6 copies of *L. licerasiae* detected by the **23S_P2** probe and average Ct of 21.57 10^6 copies of *L. wolffii* detected by the **23S_P2** probe. The same concentration of *L. biflexa* resulted in an average Ct of 38.77 by the **23S_P2** probe.

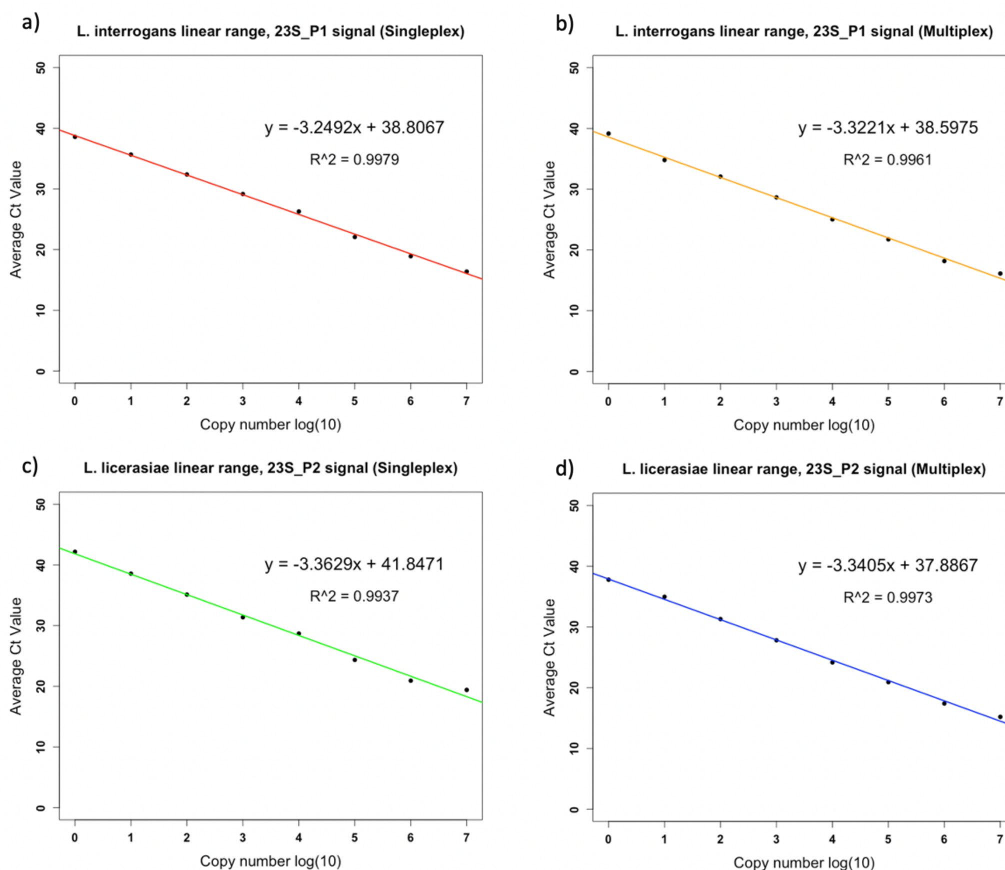
Table 3. Comparison of amplification of P1, P2, and S1 species by the *23S*-based multiplex assay under different melting temperatures. Dilutions of two representative species from each clade were tested in duplicates using **23S_P1** probe and **23S_P2** probe. Duplicate Ct values were averaged and displayed for each parameter.

		Tm = 52		Tm = 57	
Clade	Species Name	Average Ct (23S_P1 probe - FAM)	Average Ct (23S_P2 probe - JOE)	Average Ct (23S_P1 probe - FAM)	Average Ct (23S_P2 probe - JOE)
P1	L. interrogans	21.66	Undetermined	21.13	Undetermined
	L. kirschneri	19.43	Undetermined	20.04	Undetermined
P2	L. licerasiae	Undetermined	20.17	Undetermined	23.17
	L. wolfii	Undetermined	21.57	Undetermined	23.31
S1	L. biflexa	Undetermined	38.77	Undetermined	37.71
	L. vanthielli	Undetermined	Undetermined	Undetermined	Undetermined

The limit of detection and quantification of the *23S*-based assay were calculated evaluated in both singleplex and multiplex. Three standard curve dilutions were performed for each probe in singleplex and multiplex. The average limit of detection and limit of quantification were calculated, and amplification of the serial dilution was quantified using a linear range (Figure 2). The average limit of detection of the 23S_P1 probe was 1.505 in the singleplex assay and 0.898

in the multiplex assay. The average limit of quantification of the 23S_P1 probe was 4.559 in the singleplex assay and 2.720 in the multiplex assay. The average limit of detection of the 23S_P2 probe was 1.248 in the singleplex assay and 3.328 in the multiplex assay. The average limit of quantification of the 23S_P2 probe was 0.927 in the singleplex assay and 2.810 in the multiplex assay.

Figure 2. Linear range of signal from serial dilution of *L. interrogans* (P1) and *L. licerasiae* (P2) using *23S*-based assay in singleplex and multiplex. The linearity of each probe is shown alongside the coefficient of determination of each reaction.



The specificity of the *lipL32*-based qPCR assay targeting P1 species was confirmed using 12 species from the P1 group. DNA extracts from each sample were diluted to 10^6 copies per 5 μL, tested in duplicates, and Ct values were extracted and averaged. To compare the cycling efficiency of this qPCR assay in comparison to the older design, the primer/probe pairs created

by Stoddard *et al*. were utilized alongside the new design, using the same DNA extraction samples[14]. The difference in average Ct values between the new design and the design by Stoddard *et al*. were calculated, with negative ΔCt values suggesting improved efficiency from the previous design (Table 4). Of the species recently discovered since the development of the previous *lipL32*-based assay, *L. stimsonii, L. alexanderi,* and *L. mayottensis* displayed most significant decreases in Ct, with ΔCt values of -25.605, -13.465, and -8.362, respectively. Undetermined signal from 5 species from the P2 group and 5 species from the S1 groups was confirmed in order to assess the specificity of the *lipL32*-based assay for P1 species and not P2 or saprophytic species.

The specificity of the ompL37-based assay for only *L. interrogans* strains was assessed using various serotypes within the *L. interrogans* species. DNA extracts of each strain were diluted to a concentration of 10^6 copies/5 μL and analyzed in duplicates, and the Ct values of duplicates were averaged (Table 5). *L. interrogans* serovars analyzed in the assay included Copenhageni, Lai, Bataviae, Autumnalis, Hebdomadis, Pomonma, Hardjo, Icterohaemorrhagiae, Canicola, Djasiman, and Manilae. The average Ct values of each *L. interrogans* serovar included in the assay displayed a mean of 20.63 Ct and standard deviation of 0.365 Ct. Given that *L. kirschneri* and *L. noguchii* had the most similar target region sequence and least number of mismatches with *L. interrogans*, we confirmed the specificity of the assay by including *L. kirschneri* and *L. noguchii* in the *ompL37* specificity assay, eventually verifying that these species resulted in an undetermined signal.

Table 4. Comparison of amplification of P1, P2, and S1 species by the *lipL32*-based assay specifically targeting all P1 species. Dilutions of two representative species from each clade were tested in duplicates using 23S_P1 probe and 23S_P2 probe. Duplicate Ct values were averaged and displayed for each parameter.

Clade	Species Name	Ct (Stoddard et al.)	Ct (New)	ΔCt
P1	L. interrogans	18.87	19.02	0.15
	L. kirschneri	18.93	18.59	-0.34
	L. noguchii	19.08	20.24	1.17
	L. santarosai	19.27	18.99	-0.27
	L. borgpetersenii	19.53	19.60	0.07
	L. alexanderi	33.21	19.74	-13.46
	L. mayottensis	28.20	19.88	-8.33
	L. alstonii	23.77	19.27	-4.50
	L. dzianensis	24.51	20.94	-3.57
	L. yasudae	23.16	19.93	-3.22
	L. barantonii	19.03	18.43	-0.60
	L. kmetyi	22.86	19.37	-3.48
	L. weilii	19.23	29.38	10.15
	L. putramalaysiae	34.80	22.58	-12.22
	L. stimsonii	Undetermined	25.60	-25.60
	L. adleri	21.41	18.43	-2.98
	L. ellisii	22.08	18.42	-3.66
	L. gomenensis	21.13	20.87	-0.26
P2	L. licerasiae	Undetermined	Undetermined	-
	L. wolfii	Undetermined	Undetermined	-
	L. fainei	Undetermined	Undetermined	-
	L. inadai	Undetermined	Undetermined	-
	L. broomii	Undetermined	Undetermined	-

Table 5. Comparison of amplification of P1, P2, and S1 species by the *ompL37*-based assay specifically targeting the *L. interrogans* specie (P1). Dilutions of eleven representative *L. interrogans* serovars were included alongside *L. kirschneri, L. noguchii, L. licerasiae*, and *L. biflexa* to ensure reaction specificity. Duplicate Ct values were averaged and displayed for each parameter.

Specie/Serovar Type	Average Cт
L. interrogans serovar Copenhageni	21.06
L. interrogans serovar Lai	20.16
L. interrogans serovar Bataviae	20.87
L. interrogans serovar Autumnalis	20.77
L. interrogans servoar Hebdomadis	20.22
L. interrogans serovar Pomona	21.04
L. interrogans serovar Hardjo	20.24
L. interrogans servoar Icterohaemorrhagiae	20.57
L. interrogans serovar Canicola	20.98
L. interrogans serovar Djasiman	20.18
L. interrogans serovar Manilae	20.84
L. kirschneri (P1 specie)	Undetermined
L. noguchii (P1 specie)	Undetermined
L. licerasiae (P2 specie)	Undetermined
L. biflexa (S1 specie)	Undetermined

DISCUSSION

Leptospirosis is the most widespread reemerging zoonosis globally and has the potential to threaten livestock supply and medical infrastructure of lower- and middle-income countries. However, there is a limited body of literature that describes the clade- and species-specific virulence and environmental survival of *Leptospira*. Thus, recent developments in diagnostic assays that can detect and differentiate between *Leptospira* species present in various clinical and environmental contexts have provided increased opportunity to investigate infection and transmission properties of *Leptospira* species[24,25]. In context of this need for improved assays that may be used in various epidemiological contexts, qPCR assays offer the potential for rapid,

sensitive, and group-specific diagnostics that are capable of discerning the specific groups of *Leptospira* that are thriving in certain environments and/or animal hosts. The increased use of qPCR-based assays could potentially improve the underreporting of leptospirosis in light of challenges in diagnostics such as cost, time, and sensitivity in lower-income settings[26]. Thus, the purpose of this study was to design and validate a series of three qPCR-based assays that specifically detect various targets for their own respective contexts.

The *23S*-based assay utilizes a multiplex design to simultaneously detect and differentiate between P1 and P2 *Leptospira* species using a single set of primers. *23S* rRNA was identified as an optimal target due to its presence in all P1 and P2 species but considerable sequence heterogeneity between P1 and P2 clades. This presented the opportunity for the development of primers capable of amplifying sequences in both P1 and P2 species, as well as probes with unique reporters capable of binding to either P1 or P2 species. We conducted *in silico* analysis and specificity validation assays that supported the sensitivity and specificity of each probe for its respective target clade. Although the probe targeting P2 *Leptospira* species resulted in slight amplification of *L. biflexa*, a species within the S1 clade, we plan to conduct further optimization studies to improve the sensitivity of this probe to P2 species. A major advantage of the *23S*-based multiplex assay is its ability to differentiate between P1 and P2 species in future studies that aim to investigate the potential virulence and prevalence of P2 species in cases of infection among humans and host vectors.

Among these studies, the *lipL32*-based assay and the *ompL37*-based assays could be used for the detection of all P1 *Leptospira* species and *L. interrogans*, respectively. Given the high specificity of these assays, as displayed through sensitivity analyses, these assays present opportunities for the development of a new method of clinical diagnosis of leptospirosis

among humans and animal vectors. However, the performance of the *lipL32*- and *ompL37*-based assays as a robust diagnostic tool should be evaluated among clinical field samples and compared to the traditional microscopic agglutination (MAT) method, the current gold standard for leptospirosis diagnosis, as well as enzyme linked immunosorbent assay (ELISA)[27,28]. Further, the ability of qPCR-based assays to quantify copies of *Leptospira* present within clinical samples of humans and animal vectors presents a further opportunity to investigate bacterial load among infected humans during the initial acute phase of infection versus the septicemic phase of infection[29].

CONCLUSION

This study presents a collection of three qPCR-based assays for the detection of and differentiation between various *Leptospira* clades for both clinical and epidemiological contexts. Following rigorous validation of the assay using *in silico* analysis and sensitivity analysis, we confirmed specific and sensitive detection of: (1) P1 and P2 *Leptospira* species using a multiplex design targeting *23S* rRNA; (2) P1 species using a *lipL32*-based design optimized from a previous study; and (3) numerous serovars within the *L. interrogans* specie by targeting *ompL37*. This collection of assays presents as a new epidemiological and potentially diagnostic tool for the specific and robust detection of *Leptospira* species in various clinical and epidemiological studies.

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