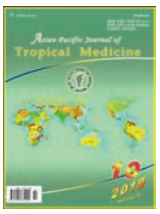

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Comparative analysis of current diagnostic PCR assays in detecting pathogenic *Leptospira* isolates from environmental samples

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ABSTRACT

Objective: To compare the efficiency of routine diagnostic PCR assays in detecting pathogenic *Leptospira* isolated from water and soils.

Methods: Seven routine assays targeting six genes (*lipL32*, *flaB*, *gyrB*, *lfb1*, *secY* and *ligB*) were evaluated and compared on the cultures of two groups of pathogenic *Leptospira* from different sources. One group included 19 described reference strains recovered from infected human or animals, and another group included 22 environmental isolates from recreational and residential sites in Malaysia. The latter have been confirmed for presence of pathogenic *Leptospira* DNA. PCR positivity or detection sensitivity of each assay was determined and compared between the two groups.

Results: Validation on reference strains showed 100.0% PCR sensitivity for all assays except *ligB*-PCR (95.0%) that failed to amplify *Leptospira interrogans* serovar Pomona. In marked contrast, there was a notable decline in sensitivity in the environmental isolates (*lipL32*-PCR, 95.5%; *flaB*-PCR, 90.9%; *gyrB*-PCR, 77.3%; *lfb1*-PCR, 59.1%; *secY*-PCRs, 40.9% G1/G2-PCR, 36.4%; *ligB*-PCR, 13.6%), implying a large genetic distance between the two groups, as well as nucleotide polymorphism among environmental isolates.

Conclusions: High proportion of false-negative PCR results suggests a need of prudent selection of primers in detecting environmental pathogenic *Leptospira*. These findings offer valuable insights on the extensive biodiversity of genus *Leptospira* and its impact on the efficacy and development of molecular detection tool.

1. Introduction

Leptospirosis is a globally widespread zoonotic infectious disease of public health concern[1] with high annual morbidity in tropical low-income countries[2]. The aetiology is highly motile spirochaete bacteria of genus *Leptospira*. Rats, rodents and domestic animals such as pigs, cattle, and dogs are common reservoirs that maintain leptospires in renal tubules and chronically excrete the bacteria in urine into environment. Humans may get infected by contact of abraded skin or mucosa membrane with the urine directly, or most often, *via* exposure to contaminated surface water or soils[3].

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Although most human leptospirosis are mild and self-resolving, the disease may causes serious damage to multiple organs including kidney, liver, lung, and sometimes fatal in severe cases.

The members of genus *Leptospira* are diverse. More than 300 serovars have been described based on antigenic relatedness as defined by the structural heterogeneity of lipopolysaccharide[4]. With the emergence of sequence-based typing method, to date *Leptospira* strains have been classified into 35 species according to whole-genome DNA homology[4,5]. The genus is also divided into three clusters based on pathogenicity and phylogeny: the parasitic pathogens, harmless free-living saprophytes, and intermediates or opportunistic pathogens.

A proper validation of analytical specificity and sensitivity of a laboratory-developed molecular diagnostics is crucial prior to its use in specimen screening practically. Numerous PCR assays targeting a number of leptospiral genes and sequences have been described for leptospirosis diagnosis and typing of *Leptospira*[6,7]. They may have been validated on a global collection of reference *Leptospira* strains or spiked clinical specimens, but PCR has yet to be developed to an extent where it is universally applied in environmental screening[8]. An accurate detection is of great importance especially during outbreak investigation where a public health investigator would trace environmental point source of transmission[9]. Recent discovery of extensive *Leptospira* biodiversity in the soils[10] and the low sensitivity of two diagnostic quantitative PCRs targeting leptospiral *lipL32* and *lfb1* genes[5] reinforces the need for re-assessment of reliability of the current assays. In view of this, this study pursues to evaluate and compare the sensitivity of selected routine PCR assays in detecting pathogenic and environmental *Leptospira* isolates.

2. Materials and methods

2.1. PCR primers

Seven primer sets targeting six different leptospiral genes, which were described in previous studies[11-17], were used in this study. The target genes include the *lipL32* gene coding for major outer membrane lipoprotein[11], *flaB* gene for flagellin[12], *gyrB* gene for DNA gyrase B[13], *lfb1* gene for putative leptospiral fibronectin-binding protein[14], *ligB* for leptospiral immunoglobulin-like protein[15], and *secY* gene for protein translocase subunit[16,17]. To differentiate the two *secY*-based PCRs, the assay described by Gravecamp and co-workers[16] was named thereafter as G1/G2-PCR.

2.2. Leptospira strains

Nineteen reference strains representing four pathogenic species of genus *Leptospira* (Table 1), obtained from WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis (the Netherlands) were used to determine the analytical sensitivity of selected PCR primers. To determine the diagnostic sensitivity in environmental isolates, twenty-two water and soil samples collected from eight amenity forests and three wet markets located in six districts in Perak, Malaysia, were used (Table 2). The presence of pathogenic *Leptospira* in culture of these samples was confirmed by PCR targeting *lipL32* gene using primers designed by Stoddard and his co-workers[18].

2.3. Collection and culture of environmental samples

Sample collection was carried out according to recommended protocols[19,20] with some modifications. Fifty millilitre of water sample was taken from waterfalls, rivers, streams, puddles, or drains by dipping a sterile tube about one foot below the water surface. Approximate 40 cm^3 of damp soil sample was taken within a 15 cm×15 cm area and 3 cm underneath the ground. Soil washing was prepared by suspending the soil sample in 10 mL sterile phosphate buffered saline and allowed to sediment for one hour.

A 5 mL aliquot of each water or soil washing sample was filtered through a nitrocellulose syringe filter (0.45 μm pore size, Pall Corp, USA). Two mL of filtrate was inoculated into 5 mL of Ellinghausen-McCullough-Johnson-Harris liquid medium (Difco™, USA) supplemented with 10% (v/v) *Leptospira* enrichment medium (Difco™, USA) and selective agent 5-fluorouracil at 200 μg/mL. The culture was then incubated at 28 ℃ for 4 weeks in dark condition.

2.4. Genomic DNA extraction and PCR amplification

Bacterial genomic DNA was extracted from 1.5 mL culture of both reference strains and environmental isolates using the EZ-10 Spin Column Bacterial Genomic DNA Mini-Preps kit (BioBasic, Canada) following the recommendations given for Gram-negative bacteria. The culture was firstly spun at 15 000×*g* for 10 min at 4 ℃ and the cell pellet was resuspended in 200 μL cold sterile Tris-EDTA buffer. The DNA extract was suspended in 50 μL of elution buffer and stored at -20 ℃.

Although the previous validation of selected PCR assays reported a flawless discrimination between the pathogenic and non-pathogenic species (intermediate and saprophytic), their analytical sensitivity was verified again using reference *Leptospira* strains before application in the environmental isolates. PCR amplification was performed in a total volume of 25 μL containing 12.5 μL of MyTaq™ mix (Bioline, UK), 400 nM of each forward and reverse primers (Integrated DNA Technologies, Singapore), and 1 μL of DNA extract from culture of reference strains, or 2 μL from sample cultures.

All amplifications were performed in a thermal cycler (MyCycler™, Bio-Rad, USA) with a programme comprising one cycle of pre-denaturation at 95 ℃ for 3 min followed by 35 cycles of amplification at 95 ℃ for 15 s, 55 ℃ for 15 s and 72 ℃ for 10 s. The amplification

Table 1. Nineteen reference *Leptospira* strains used in this study.

Serovar	Serogroup	Strain	Species
Australis	Australis	Ballico	*Leptospira interrogans*
Autumnalis	Autumnalis	Akiyami A	*Leptospira interrogans*
Ballum	Ballum	Mus 127	*Leptospira borgpetersenii*
Bataviae	Bataviae	Swart	*Leptospira interrogans*
Canicola	Canicola	Hond Utrecht IV	*Leptospira interrogans*
Celledoni	Celledoni	Celledoni	*Leptospira weilii*
Copenhageni	Icterohaemorrhagiae	M 20	*Leptospira interrogans*
Djasiman	Djasiman	Djasiman	*Leptospira interrogans*
Grippotyphosa type Moskva	Grippotyphosa	Moskva V	*Leptospira kirschneri*
Hardjo type Bovis	Sejroe	Sponselee	*Leptospira borgpetersenii*
Hardjo type Prajitno	Sejroe	Hardjoprajitno	*Leptospira interrogans*
Hebdomadis	Hebdomadis	Hebdomadis	*Leptospira interrogans*
Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	*Leptospira interrogans*
Javanica	Javanica	Veldrat Batavia 46	*Leptospira borgpetersenii*
Lai type Langkawi	Icterohaemorrhagiae	Langkawi	*Leptospira interrogans*
Lai	Icterohaemorrhagiae	Lai	*Leptospira interrogans*
Pomona	Pomona	Pomona	*Leptospira interrogans*
Pyrogenes	Pyrogenes	Salinem	*Leptospira interrogans*
Tarassovi	Tarassovi	Perepelitsin	*Leptospira borgpetersenii*

Table 2. Source of pathogenic *Leptospira* isolates from environmental samples.

Sampling site	Districts	Coordinates	*Leptospira* isolate	Source
Amenity forest				
LK	Batang Padang	4.3003°N, 101.2545°E	LK 3	Waterfall water
			LK 7	Waterfall water
BL	Larut, Matang & Selama	4.8618°N, 100.7610°E	BL 7	Stream bank soil
UKE	Kuala Kangsar	4.6894°N, 100.8860°E	UKE 3	River water
UKI	Kinta	4.6697°N, 101.1984°E	UKI 2	River water
			UKI 7	River water
KS	Kinta	4.3003°N, 101.2545°E	KS 1	Stream water
KF	Kampar	4.4668°N, 101.2230°E	KF 1	Stream water
			KF 6	Forest soil
			KF 10	Waterfall water
			KF 11	Waterfall water
BB	Kampar	4.3078°N, 101.1686°E	BB 1	Waterfall bank soil
			BB 3	Waterfall bank soil
			BB 9	Waterfall bank soil
			BB 12	Waterfall bank soil
UC	Kinta	4.7096°N, 101.0686°E	UC 1	Stream bank soil
			UC 3	Stream water
			UC 4	Stream bank soil
			UC 8	Stream water
Wet market				
PP	Kinta	4.5761°N, 101.0871°E	PP 9	Market drainage water
MG	Kinta	4.5668°N, 101.0465°E	MG 9	Market drainage water
KM	Kampar	4.3157°N, 101.1501°E	KM 9	Market drainage soil

was completed with a final extension at 72 ℃ for 10 min. A non-template control, in which DNA template was replaced by sterile distilled water, was included in each reaction. Reactions were repeated in duplicates. Five μL of amplicon was visualised using 1.8% (w/v) Tris-Acetate-EDTA (TAE)-agarose gel electrophoresis at 150 mA for 35 min. All gels were prepared with HealthView™ Nucleic Acid Stain (Genomics, Taiwan) according to manufacturer's recommendation.

2.5. Sequencing

The selected PCRs were also used to amplify *Leptospira* (*L.*) *interrogans* serovar Icterohaemorrhagiae to confirm if target gene has been successfully amplified. Amplicons were purified and sequenced by a DNA sequencing service provider (1st BASE, Selangor, Malaysia) which uses Applied Biosystems genetic analyzer platform and BigDye® Terminator v3.1 cycle sequencing kit chemistry. Chromatograms were assembled and analysed in Sequence Scanner software version 2.0 (Applied Biosystems).

2.6. Determination of lowest limit of detection (LLOD)

The LLOD of each PCR primer set was also determined using serial dilutions of DNA of *L. interrogans* serovar Canicola ranging

from 10 000 to 10 pg per reaction. The concentration of DNA template was firstly adjusted to 10 ng/μL, and then the 10-fold serial dilutions were prepared. One μL of each DNA dilution was then added into corresponding reaction. The lowest concentration of DNA that showed a visible band in PCR was taken as the LLOD.

3. Results

3.1. Amplicon and detection limit of PCR assays

Each assay has successfully yielded single amplicon corresponding to the expected size and a correct gene target as confirmed by sequencing. In determining the LLOD, the amplicon was detectable for all PCRs using the least DNA template (10 pg) containing an average of 2 000 genome equivalents per reaction [based on the genome size of *L. interrogans* strain Fiocruz L1-130 (4.6 Mb); 1 genome=5 fg gDNA], except the G1/G2-PCR and *ligB*-PCR of which the yields were 10- and 100-fold lesser, respectively (Figure 1). Two μL of 50 mM $MgCl_2$ was added for *ligB*-PCR per reaction to increase the amplicon yields.

3.2. Validation of PCR assays on reference Leptospira strains

Results showed a 100% analytical sensitivity or true positive rates for all PCR assays but a slightly lower rate (95%) for *ligB*-PCR (Table 3). Obtaining negative result consistently despite the increased amount of DNA template (up to 8.3 ng) has confirmed the unsuccessful amplification of *L. interrogans* serovar Pomona by *ligB*-PCR in this present study.

3.3. Use of PCR assays on environmental Leptospira isolates

In contrast, the PCR amplification of environmental isolates revealed a range of lower and varied sensitivities (13.6% to 95.5%) (Table 3). *lipL32*-PCR showed the highest sensitivity (95.5%), followed by *flaB*-, *gyrB*- and *lfb1*-PCR, whereas *secY*-PCR, G1/G2-PCR and *ligB*-PCR detected less than 50.0% of samples. Comparison between the two distinct groups of pathogenic *Leptospira* showed a notably decrease in sensitivity (4.5% to 86.4%) and high percentage of false-negative PCR reactions in the environmental isolates (40.9%, 63/154) in relation to the host-associated strains (0.8%, 1/133) (Table 3). This finding thus raised a concern on the accuracy of current PCR assays in detecting environmental isolates.

4. Discussion

Although the original studies[11-17] reported a faultless detection of all described pathogenic species of genus *Leptospira*, the reproducibility of the selected PCR primers was assessed. The 100% analytical sensitivity determined in the present study was well founded since similar reference strains were tested. However, a false-negative reaction occurred unexpectedly where *L. interrogans* serovar Pomona could not be detected by *ligB*-PCR as opposed to the previous study[15]. The template DNA amount used in amplification (8 300 pg) should not be an issue with regards to the LLOD of *ligB*-PCR determined in the present study (1 000 pg per reaction). The discrepancy raises a concern with the result inaccuracy concerning this assay to be used for diagnosis and detection of leptospires. As a result, there is a need to fully verify and improve the robustness and efficacy of *ligB*-PCR in detecting all pathogenic species.

Following the validation, similar primer sets were used to amplify pathogenic *Leptospira* of unknown type isolated from environmental water and soils. All PCRs are supposedly positive regardless of target gene given the presence of DNA of pathogenic *Leptospira* in these samples. Nonetheless, none of the primer sets could detect all environmental isolates, even the one targeting *lipL32*. The large total number of false-negative reactions is a matter of great concern, implying the shortcomings of current PCR assays particularly in detecting environmental isolates. Also, the PCR sensitivities

Table 3. PCR sensitivity determined in reference strains and environmental isolates of pathogenic *Leptospira* species.

PCR target gene/ primer	Reference strains			Environmental isolates			Decrease in sensitivity (%)
	True positive	False negative	Sensitivity (%)	True positive	False negative	Sensitivity (%)	
lipL32	19	0	100.0	21	1	95.5	4.5
flaB	19	0	100.0	20	2	90.9	9.1
gyrB	19	0	100.0	17	5	77.3	22.7
lfb1	19	0	100.0	13	9	59.1	40.9
secY	19	0	100.0	9	13	40.9	59.1
G1/G2	19	0	100.0	8	14	36.4	63.6
ligB	18	1	95.0	3	19	13.6	86.4
Total	132	1	-	96	72	-	-

Nineteen reference strains and 22 environmental isolates were used to determine and compare the sensitivity of seven pathogen-specific PCRs targeting six different genes. Decrease in sensitivity for each PCR assay was also determined by subtracting the sensitivity for reference *Leptospira* strains from that for environmental isolates.

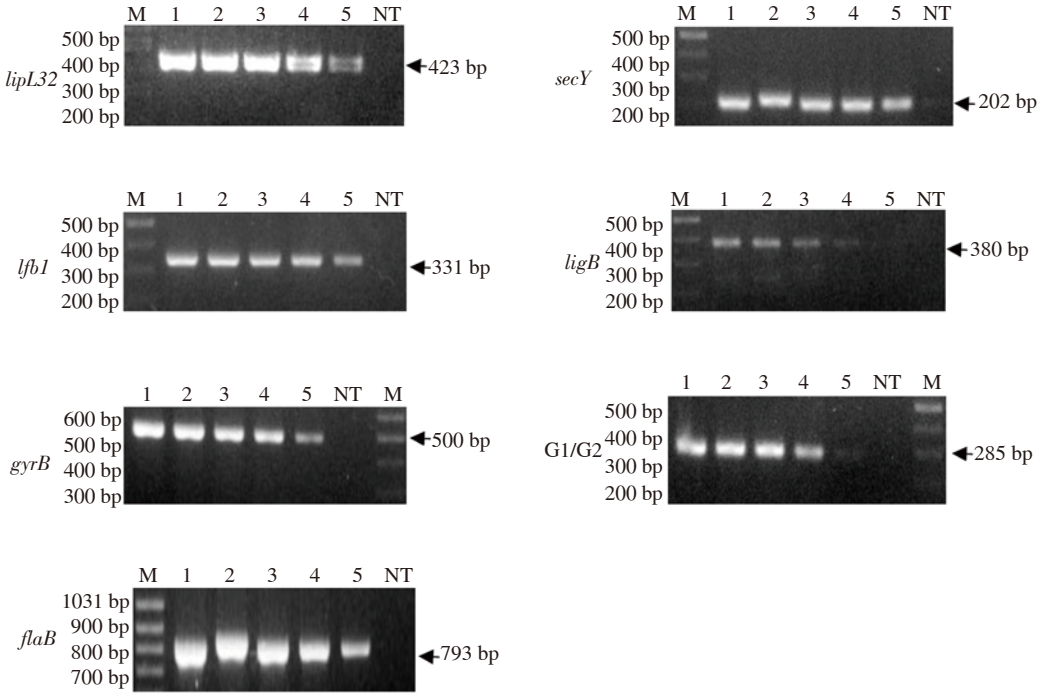

Figure 1. Comparison of lowest detection limit among PCRs. Lanes 1-5: varied DNA template amount added per reaction from 20 000 pg (lane 1), 10 000 pg (lane 2), 1 000 pg (lane 3), 100 pg (lane 4) to 10 pg (lane 5); M, MassRuler™ DNA ladder mix; NT, non-template control.

determined in environmental isolates were much lower and widely varied to that in the host-sourced strains, suggesting genetic distance and divergence between the two groups.

Absence of amplification was likely attributed to mismatches between template and primer as a result of sequence variation. Impact of single-nucleotide polymorphisms on PCR detection of *Leptospira* was firstly reported by Bourhy and his co-workers[21] who failed to amplify soil-derived *L. kmetyi* using *secY*-PCR. The sequencing reveals a total of five mismatches, two in the forward primer and three in the reverse primer, among which one is located two bases from the 3' end of forward primer[21]. Having such a frequency and position of mismatches would block a reaction completely for lacking primer extension[22]. Overall, our findings suggest a large genomic plasticity and unexplored biodiversity in genus *Leptospira* and are in agreement with Thibeaux and his co-workers[5] who also reported low detection sensitivity of quantitative PCRs as a result of polymorphisms in *lipL32* and *lfb1* genes[10].

Although the environmental samples have been previously detected positive for pathogenic *Leptospira*, a question has been in dispute if other genetic markers, besides *lipL32*, are also conserved among these isolates. Another question worth exploring is about the sensitivity of different primer sets targeting similar gene. The PCR primer sets of study amplify a broad range of major leptospiral genes, including three pathogen-restricted genes (*lipL32*, *ligB*, *lfb1*) and three housekeeping genes (*secY*, *flaB*, *gyrB*), and are routinely used in reference laboratories globally and surveillance studies[7]. It is postulated that the PCR sensitivity may correlate with the degree of sequence variability of the target gene. The higher polymorphism, the more mismatches and hence blocked amplification[22]. In this study, the sensitivities of PCRs targeting *secY* and *ligB* were notably lower than that of *lipL32*, suggesting a greater sequence diversity in the former. Further evidence supporting this was the higher percentage of variable nucleotide sites in *secY* than in *rrs*, *lipL32* and *lipL41* during an assessment of gene loci for genotyping by multilocus sequence typing method[23]. However, such a high diversity may be a major obstacle in development of sequence-based molecular diagnostics by the difficulty in identifying conserved sequences, as exemplified by the *lig* genes[24].

It is also important to highlight that G1/G2- and *ligB*-PCR need more DNA template for a detectable yield of amplicon given their LLOD was one to two log higher than other PCRs. Also, the detection sensitivity is unlikely dependent with genetic marker for the LLOD determined for two PCRs targeting similar gene (*secY*) were not similar. In the light of these findings, it is interesting to further explore the impact of LLOD on efficacy of a primer set.

In this present study, *lipL32*- and *flaB*-PCR showed the highest sensitivity. Pathogen-restricted *lipL32* gene was most often targeted in development of molecular diagnostics[25] and used in environmental surveillance[26-28]. Findings of other studies in a variety of sample types support the higher sensitivity of PCR targeting *lipL32* to other genetic markers. For example, *lipL32*-PCR was found to be more sensitive than *ompL1*-PCR in human and bovine serum samples[29] and in buffalo kidney samples as compared to G1/G2-primed *secY*-PCR[30]. On the other hand, findings of some

studies rather support better performance of G1/G2-PCR, such as in detecting *L. borgpetersenii* Hardjo-bovis in bovine urine samples in comparison to *rrs*-PCR[31], in spiked human blood and urine samples than LP1/LP2 primers[32], and in environmental sample than *lipL32*-PCR[33]. Due to lacking of well-ground and consistent demonstration of high detection sensitivity of a particular assay, it is difficult to make a valid conclusion on which genetic marker or PCR primers is most sensitive for accurate detection. The underlying cause for the variability in different circumstances is unclear but it may be dependent on localised *Leptospira* biodiversity in a particular region or source.

Most of the current diagnostic PCRs take the important conserved sequences in the genome of *L. interrogans*, or among a panel of *Leptospira* strains representing global source, as the model target in designing primers. Its analytical specificity and sensitivity are validated experimentally using known relevant strains, followed by the spiked specimens for diagnostic applicability. The primers may not complement to all strains considering the overlooked biodiversity of the genus *Leptospira*[5]. The high specificity of PCR technique would compromise the sensitivity, especially in the case of rare or atypical strains isolated from the environment[10].

The G1/G2-PCR has been widely used in Malaysia for environmental epidemiological studies[33-36] besides the *lipL32*-PCR[26-28]. Because of the comparatively low sensitivity of G1/G2-PCR as determined in this present study, it is of great concern if the occurrence of leptospiral DNA reported (0.7%-7.5%)[33-36] was underrated. Moreover, there is one shortcoming of G1/G2 primers for its incapability in detecting *L. kirschneri*[16]. To ensure accurate detection of all pathogenic species, another primer set (bim-based B64-I/B64-Ⅱ) that specifically detect *L. kirschneri*[16] should be included. Overall, our findings implies that a comprehensive validation of a laboratory-developed PCR assay is important before its use in field setting in view of the large biodiversity of genus *Leptospira*. An earlier environmental epidemiological study reported a rich genotype diversity of *Leptospira* in Malaysia by typing the pathogenic *Leptospira* isolated from recreational lakes and wet markets using pulse-field gel electrophoresis[35].

In conclusion, recent works as well as our findings bring the reliability into question of most diagnostic PCRs, particularly *secY*- and *ligB*-PCRs, in the context of environmental *Leptospira*. Our findings also suggest a large genetic polymorphism and biodiversity in environmental *Leptospira*, which may be further verified using whole-genome sequences and a comprehensive identification tool. There is also a growing need to continue identify conserved PCR target sequence to improve the relevance of sequence-based molecular diagnostics.

Conflict of interest statement

We declare that we have no conflict of interest.

Authors' contribution

Y.M.L. developed the concepts, designed the experimental study, acquired the data and prepared the manuscript. Y.M.L., C.H.Y. and O.H.K.A. performed the data analysis. All authors reviewed and edited the manuscript.

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