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Clinical evaluation of an in-house probe-based real-time PCR assay for early diagnosis of leptospirosis compared with Microscopic Agglutination Test

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ABSTRACT

Introduction: TaqMan probe-based real-time PCR assay targeting the lipL32 gene offer higher specificity and accuracy than conventional PCR for leptospirosis diagnosis. However, it has not been implemented in Sri Lanka.

Methods: An in-house real-time PCR assay targeting lipL32 gene of pathogenic *Leptospira* species was developed and validated with known DNA extracts. PCR assay was performed for whole blood of 30 clinically suspicious patients within the first five days of illness and compared with Microscopic Agglutination Test (MAT), performed after day six with titers ≥ 1 : 320 considered positive. Haematological and biochemical results were retrieved.

Results: Of 30 patients, 16 (53.3%) were confirmed positive: 14 by MAT alone and 2 by both PCR and MAT. DNA from *Leptospira interrogans* serovar Icterohaemorrhagiae was consistently amplified across six independent runs with an average CT value of 12. The two clinical PCR-positive samples showed CT values of 28 and 30. Compared with MAT, the PCR assay had a diagnostic sensitivity of 13.3% and specificity of 100%. Haematological findings included neutrophilia (>70%) in 86.7%, lymphopenia (<20%) in 90%, and thrombocytopenia (<100×10⁹/L) in 96.7%. All patients had elevated C-reactive protein (>5 mg/L). Most had raised AST levels (>35 U/L), while ALT and serum creatinine were largely normal.

Conclusions: The newly designed primers and probes successfully detected *Leptospira* DNA positive controls and two clinical samples. Hence, further validation with a larger sample size may enhance the assay's sensitivity and clinical utility.

Keywords: *Leptospirosis, MAT, Probe based, Real time PCR.*

Introduction

Leptospirosis/ Weil disease is a widespread and potentially fatal zoonotic infectious disease of animals/humans. It is the most common zoonotic infection in the world. It is caused by the pathogenic bacterium called *Leptospira,* that are easily transmitted from infected animals through their urine, either directly or through infected soil or water (1, 2).

According to the Leptospirosis Burden Epidemiology Reference Group (LERG), the risk of spreading the leptospirosis disease increases due

to rainfall, flooding, open sewers, crowding, populace, animal contacts, and poor sanitation (3). Leptospirosis was first considered as an occupational disease. Men are more likely to be infected with leptospirosis as they are more prone to occupational exposure in outdoor settings (4).

In the septicemic phase, pathogenic *Leptospira* are present in the blood and cerebrospinal fluid (CSF). The characteristic feature of this phase is febrile illness and it may last for 3 to 10 days (5). In the second phase which is called as immune phase, antibodies are released to the blood against *Leptospira* present in the blood. This phase usually occurs in the second week and lasts 4 to 30 days. This phase is accompanied by the elimination of the *Leptospira* from the blood and excretion in the urine (6).

Laboratory investigations are critical to confirm leptospirosis as clinical manifestations can mimic other febrile illnesses. Supportive diagnostic tests or screening tests to detect leptospirosis are IgM-based commercial assays such as ELISA IgM, Immuno-DOT, and Lateral flow tests (3).

Out of the confirmatory diagnostic tests, Microscopic Agglutination Test (MAT) is the gold standard test currently used to detect the leptospirosis based on serology. As anti-leptospira antibodies of IgM and IgG usually become detectable from 5 to 7 days after the onset of illness, MAT can confirm the disease at the late acute phase only. Panel of live *Leptospira* belonging to different serovars must be used as antigens in the MAT unless antibodies to the serovar missing from the panel may not be detected and serodiagnosis may give inaccurate or false-negative results. The major advantage of the MAT is its high specificity and disadvantages are the need for facilities to culture and maintain a representative panel of live *Leptospira* comprising all causative strains and the technically demanding and time-consuming process (7).

Thus, to start the most optimal treatment at the most effective time point, the availability of an accurate diagnostic test to detect the disease in the early acute phase is essential. The PCR technique, which can detect the DNA of pathogenic *Leptospira* present in the blood in the first 5 to 10 days, may be the best for such an early diagnosis with high sensitivity and specificity. Another advantage of PCR is that it enables quantitative monitoring of *Leptospira* cells, it gives opportunity to monitor treatment efficacy. Hence, the PCR assay will be more beneficial than MAT (8). At present, a few reference laboratories in Sri Lanka perform leptospirosis PCR assay. The PCR methods used there are conventional PCR assay and dye-based (SYBR green) real-time PCR assay. These two methods are generally simple, less expensive, and do not require specific probes for each target, The SYBR green technique is less specific than Taq Man probe-based methods as the dye can bind to any double-stranded DNA and needs careful optimization to avoid non-specific signals. The TaqMan probe-based method utilizes sequence specific probes that bind to specific sequence in the target DNA. As such, there is an urgent need for rapid and reliable diagnostic tools in Sri Lanka. We developed an in-house TaqMan probe-based real-time PCR assay targeting the lipL32 gene, a specific marker in pathogenic *Leptospira* species. This study aimed to describe haematological and biochemical features of selected group of patients suspected to have leptospirosis and to clinically evaluate the performance of the in-house PCR assay in comparison with MAT.

Methods

Study design and setting

A cross-sectional descriptive study was carried out from June to September 2024 at the National Hospital Galle (NHG), a tertiary referral hospital in Southern Sri Lanka. Thirty consecutive in-patients presenting with suspected leptospirosis, as assessed by ward physicians, were recruited. Ethical approval was obtained from the Ethics Review Committee of the Faculty of Allied Health Sciences, University of Ruhuna (Ref. 2023.12.364). Written informed consent was obtained from all participants.

Inclusion and exclusion criteria

Patients were eligible if they presented with fever and headache accompanied by at least one additional symptom, including myalgia, oliguria or polyuria, conjunctival suffusion, chest pain, or dyspnea. Patients with recent occupational or

environmental exposure such as farming, paddy field work, or flood water contact were prioritised. Exclusion criteria included traumatic or post-operative fever, confirmed alternative infections such as dengue or malaria, or discharge before collection of the second serum sample for MAT.

Sample size justification

Sample size was calculated using a diagnostic test evaluation formula, assuming 50% prevalence of leptospirosis among clinically suspected patients, with a 95% confidence level. This yielded a minimum requirement of 30 patients for preliminary evaluation (9).

Sample collection

Two types of samples were collected. EDTA whole blood was obtained within the first five days of illness for DNA extraction and PCR analysis. Serum was collected between day 6 and day 7 of illness for MAT testing. All patients remained in hospital until the second sample was collected.

DNA extraction and PCR protocol

DNA was extracted using the Promega ReliaPrep Blood gDNA Miniprep System. This system isolates gDNA using cellulose as the binding matrix under specific conditions. The in-house PCR assay was designed to amplify a segment of the lipL32 gene. Primers and probe sequences are listed in Table 1. Each reaction had a total volume of 23 µL, consisting of GoTaq Probe qPCR Master Mix, primers, probe, and 5 µL of extracted DNA. RNaseP gene served as internal control. Components, their volumes and final concentrations used for the reaction mixture were showed in Table 2 and Table 3.

Amplification and fluorescence detection were performed on QuantStudio5DX with the following thermal profile. The programme contains 45 cycles, each cycle consisting of a reverse transcription step at 45^0C for 15 minutes, initial denaturation at 95^0C for 2 minutes, and another denaturation step at 95^0C for 3 seconds and detection and annealing at 55^0C for 30 seconds. Fluorescence was detected at the annealing step using Fam as the dye. Positive controls consisted of DNA from *Leptospira interrogans* serovar Icterohaemorrhagiae culture; negative controls used nuclease-free water. Positive and negative controls were tested in six independent runs to confirm reproducibility.

MAT procedure

MAT was performed at the Medical Research Institute (MRI), Colombo, using a panel of locally relevant *Leptospira* serovars. A MAT titer ≥ 1 : 320 was considered positive. Paired sera were not available due to practical challenges of patient follow-up.

Data collection and analysis

Demographic data, occupational exposures, clinical features, Haematological indices (platelet count, WBC, RBC, haemoglobin, hematocrit, MCV, neutrophil %, lymphocyte %, and biochemical markers (AST, ALT, creatinine, CRP) were retrieved from patient records. Abnormal values were defined according to the local reference ranges. Data were analyzed with SPSS v26. Diagnostic sensitivity and specificity of PCR were calculated using MAT as the reference standard.

Table 1: Primer and probe sequences used in the in-house qPCR assay

Target gene	Name	Sequence (5' → 3')	Label/ Notes
lipL32	LipL32 - 45F	5′ – AAg CAT TAC CgC TTg Tgg Tg - 3'	Forward primer
lipL32	LipL32 - 286R	5′ –gAACTCCCATTTCAgCgATT - 3'	Reverse primer
lipL32	Probe	/56-FAM/AA AGC CAGG/ ZEN/ A CAA GCG CCG/ 3IABkFQ/	TaqMan probe

Table 2: In-house modified reaction mixture preparation protocol for target (leptospiral target) assay

Component	1×	Final concentration
Nuclease free water	3.1 µL	-
qPCR Master mix	10 µL	1×
RT Mix	0.4 µL	10 µM
Primer F	1.5 µL	10 µM
Primer R	1.5 µL	10 µM
Probe	1.5 µL	100 – 300 nM
Total mixture	18 µL	-
Extracted DNA	5 µL	≤ 250 ng
Total	23 µL	-

qPCR: quantitative polymerase chain reaction; RT: Reverse Transcriptase; DNA: Deoxyribo Nucleic

Table 3: In-house modified reaction mixture preparation protocol for internal control assay

Component	1×	Final concentration
Nuclease free water	6.1 µL	-
qPCR Master mix	10 µL	1×
RT mix	0.4 µL	10 µM
Probe primer mix	1.5 µL	10 µM
Total mixture	18 µL	-
Extracted DNA	5 µL	≤ 250 ng
Total[a]	23 µL	-

qPCR: quantitative polymerase chain reaction; RT: Reverse Transcriptase; DNA: Deoxyribo Nucleic Acid

Results

Patient demographic and exposure risks

A total of 30 clinically suspected patients with leptospirosis were enrolled. Among them 86.7% were male. The mean age of the suspected cases was 42±20 years. In terms of occupation, majority (70%) of the suspected patients involved in outdoor activities. Exposure to animals could be a potential risk factor for the disease. The presence of rats in home, work place or any other exposed environment was found in 43.3% of the patients. When consider the environmental factors, 40% had exposed to agricultural lands.

Haematological and biochemical findings

Haematological profile and bio chemical profile of clinically suspected leptospirosis cases was studied at the time of hospital admission and after a week of onset of symptoms. Of the suspected patients 86.7% had neutrophilia (>70%), 90% had lymphopenia (<20%), 96.67% had thrombocytopenia (<100×10^9/L) on admission. All participants had elevated C-reactive protein (>5 mg/L) and 63.3% of patients had elevated AST level (>35U/L). However, ALT (0-45U/L) and serum creatinine (74-110 µmol/L) levels were within reference range in 66.7% and 53.3% patients respectively (Table 4).

Table 4: Haematological profile of study population

Parameter	Profile at hospital admission			Profile after a week of symptoms			Median± range at hospital admission	Median± range after week of symptoms	*p* value
	Low levels	Within reference range	High levels	Low levels	Within reference range	High levels			
Platelet count (10^9/L)	96.67%	3.3%	-	36.7%	60%	3.3%	103.50± 294.00	123.00± 299.00	0.03
RBC count (10^{12}/L)	-	96.67%	3.3%	6.7%	93.3%	-	4.30± 2.61	4.04± 1.90	<0.01
Haemoglobin level (g/dL)	16.7%	80%	3.3%	20%	80%	-	12.55± 7. 00	11.70± 5.30	<0.01
HCT %	53.3%	46.7%	-	6.7%	33.3%	-	36.55± 19.50	33.55± 17.00	<0.01
MCV (fL)	16.7%	83.3%	-	20%	80%	-	84.54± 27.10	84.10± 26.40	0.08
WBC count (10^9/L)	-	56.7%	43.3%	-	63.3%	36.7%	9.27± 15.61	7.86± 21.70	0.16
Neutrophil %	3.3%	10%	86.7%	30%	36.7%	33.3%	85.10± 59.00	65.45± 63.30	<0.01
Lymphocyte %	90%	6.7%	3.3%	43.3%	36.7%	20%	9.60± 44.40	23.45± 48.60	<0.01

SD: Standard Deviation; RBC: Red Blood Cell; HCT: Hematocrit; MCV: Mean Cell Volume; WBC: White Blood Cell

Diagnostic outcomes

Out of the total, 16 (53.3%) patients were confirmed to have leptospirosis; 14 only by MAT, and 2 patients both by PCR and MAT. The MAT titer of ≥ 1 : 320 was considered as positive which is the cutoff specified by the MRI (Table 5). Even though a large majority of patients had low platelet count, all the patients had elevated CRP levels. Significant differences were observed in platelet count (p= 0.022) and CRP level (p=0.006) among leptospirosis confirmed and non-confirmed cases at the time of hospital admission.

Accuracy and precision of the in-house PCR assay were determined using Leptospira DNA extracts from *Leptospira interrogans* serovar Icterohaemorrhagiae cultures having DNA concentration 13.2 ng/µL and 12.6 ng/µL respectively.

In terms of accuracy, these two extracts were tested using new in-house PCR technique, total of 6 independent runs and each obtained amplification curves at average cycle threshold (CT) value 12. Precision was determined using SYBR green dye based real time PCR technique and obtained positive results.

Samples were run targeting both lipL32 gene (Leptospira target) and RNase P gene (internal control). Two samples showed amplification for Leptospira target (lipL32 gene) and obtained CT value 28 and 30 respectively. All the 30 samples showed amplification for internal control ensuring sufficient DNA extraction. Diagnostic sensitivity and diagnostic specificity values were determined comparing with MAT which is the gold standard test to detect leptospirosis and they were 13.33% and 100% respectively.

Table 5: MAT and PCR results of leptospirosis confirmed cases

Leptospirosis confirmed cases by MAT	MAT titer	PCR results	CT value	Days of fever when samples are collected for MAT	Days of fever when samples are collected for PCR
1	1280	Positive	28	7	5
2	640	Negative	-	7	5
3	640	Negative	-	7	5
4	640	Negative	-	7	5
5	2560	Negative	-	7	5
6	320	Negative	-	7	5
7	1280	Positive	30	7	5
8	5120	Negative	-	7	5
9	10240	Negative	-	6	5
10	2560	Negative	-	7	5
11	2560	Negative	-	7	5
12	640	Negative	-	6	5
13	2560	Negative	-	7	3
14	640	Negative	-	7	5
15	640	Negative	-	7	5
16	1280	Negative	-	7	5

MAT; Microscopic Agglutination Test; PCR: Polymerase Chain Reaction; CT: Cycle Threshold

Discussion

The present study was conducted to detect the leptospiral DNA by in-house TaqMan probe-based real time PCR method and to provide a preliminary clinical evaluation of the assay by comparing with MAT which is the gold standard test to diagnose leptospirosis, enrolling clinically suspected patients. Probe-based PCR method utilizes sequence specific probes that bind to specific sequence in the target DNA, it has very high sensitivity and specificity and provide better discrimination between target and non-target sequences than other methods available in Sri Lanka. Hence in-house TaqMan probe-based real time PCR assay was developed using newly designed probes and primers.

Although the assay showed perfect specificity, sensitivity was low, identifying only two positive cases compared with 16 confirmed by MAT. These findings emphasize both potential and the limitations of probe-based PCR in a real-world clinical setting (10, 11). Several factors may explain the low sensitivity observed. First, the timing of blood collection was within the first five days of fever, a period expected to correspond to the bacteraemic phase. In leptospirosis, the incubation period from exposure to onset of symptoms average from 7 to 12 days though it can be as 3 days or as long as a month (1). *Leptospira* can be present in the blood during the incubation period even if the person is asymptomatic or has not yet developed noticeable symptoms. Hence, variability in host-pathogen dynamics means that some patients may have already cleared organisms from the bloodstream by the time of sampling. Second, many suspected leptospirosis patients receive empirical antibiotics upon hospital admission, which could limit the detection threshold before or after admission. Antibiotic utilization before admission was not thoroughly recorded, which is the notable limitation of this study.

MAT, despite being the reference standard, has significant drawbacks (12). High antibody titer depends on several factors including stage of infection, duration of illness, previous exposure, nature of immune response and epidemiological patterns. Furthermore, MAT may show high titers if there is a cross-reactivity with antibodies from infections with other serovars or related pathogens, but normally in clinical setup high antibody titer indicates the severity of infection (13, 14). Antibodies can be generated against bacterial components, toxins or other molecular signals released during infection without needing to find pathogen in blood. The lack of paired sera in the study may also have underestimated true MAT positivity, as single samples can yield false negatives in early infection.

MAT positivity is based on the specified cutoff, however some studies emphasized that high endemic countries like Sri Lanka the cutoff should be ≥ 1 : 800 (15, 16). MAT titer of PCR positive samples was 1 : 1280. When consider the cutoff as ≥ 1 : 800 PCR negative and MAT positive 7 samples were found and their titer range was 1:10 280-1 :2560. Among PCR positive two patients, one is exposed to flood and another involved in gardening at pre infection period. Further, both of them involve in occupations with outdoor activities. When consider their Haematological and bio chemical parameters they were correlated with previously published findings but could not find considerable variations than other MAT positive and PCR negative patients' parameters (17).

When comparing our findings with other studies, probe-based PCR assays generally report sensitivities ranging from 40% to 70% and specificities above 90%. Despite this, finding of successful detection in positive controls and two clinical cases indicates that the assay is technically satisfactory however, further optimisation and validation is required.

Even though MAT is the gold standard test to diagnose leptospirosis, MAT positivity may not due to acute leptospirosis but due to any other reason that can trigger antibody production (18). Moreover, out of the suspected patients nearly half of the patients were positive for MAT. That may be due to the locally and regionally relevant *Leptospira* serovars not representing the tested MAT panel. Hence, determination of *Leptospira* seroprevalence in Southern region and adjusting diagnostic MAT cutoff according to the epidemiology patterns and distribution of *Leptospira* serovar are essential for the management and the prevention of disease outbreaks. Further, to determine the cutoff, baseline antibody levels have to be established in the general population seasonally, regionally and according to the serovar and threshold has to be defined that

can reflect active leptospirosis with a suggestive clinical presentation, to avoid false conclusions (19).

Haematological and biochemical findings in our cohort were consistent with previous reports. Thrombocytopenia and lymphopenia are common in acute leptospirosis and often overlap with other febrile illnesses. Elevated CRP and AST reflect systemic inflammation and hepatic involvement, both frequently described in leptospirosis patients. These laboratory findings could not be used as a diagnostic tool, however, may support clinical suspicion and guide empirical treatment.

This study demonstrates several limitations including small sample size which reduced the precision of diagnostic accuracy measurements and lack of paired sera for MAT interpretation. Additionally, antibiotic exposure prior to sampling was not documented. The study was conducted in a single center over a short period of time. Hence, future studies can be expanded for wider population and longer time durations including several disease outbreaks across different regions of Sri Lanka. To fully realize the PCR assay's potential further research is needed to investigate the sensitivity and specificity as well as to evaluate its performance to detect various *Leptospira* serovars. Moreover, the impact of antibiotic therapy on PCR performance and the feasibility of implementing the assay in government hospitals need to be determined. Optimization of sample collection timing, exploration of urine as an alternative specimen for PCR and collection of serial blood and urine samples may enhance the diagnostic sensitivity. By addressing these study gaps, the probe-based PCR assay can be introduced as a supplementary diagnostic tool for improving leptospirosis diagnosis and treatment outcomes in Sri Lanka.

Conclusions

The in-house probe-based real-time PCR assay targeting the lipL32 gene demonstrated high specificity and low sensitivity when compared with MAT in diagnosing clinically suspected leptospirosis patients. However, the PCR assay demonstrates considerable potential as a complementary diagnostic tool, particularly for early detection before the development of detectable antibody titers. Based on the current findings, the newly designed primers and probes successfully detected *Leptospira* DNA positive controls and two clinical samples ensuring technical permormance of the assay. Further validation with a larger sample size, optimization of sample collection timing and specimen type, focusing empirical antibiotic treatment outcomes for PCR assay and determination of sensitivity and specificity using different serovars may enhance the assay' performance and clinical utility.

The authors have no conflicts of interest to declare.

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