Accepted Manuscript

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PII: S0003-2670(15)01374-4

DOI: 10.1016/j.aca.2015.11.015

Reference: ACA 234254

To appear in: *Analytica Chimica Acta*

Received Date: 23 August 2015

Revised Date: 25 October 2015

Accepted Date: 5 November 2015

Please cite this article as: A.B. Nurul Najian, E.A.R. Engku Nur Syafirah, N. Ismail, M. Mohamed, C.Y. Yean, Development of Multiplex Loop Mediated Isothermal Amplification (m-LAMP) Label-based Gold Nanoparticles Lateral Flow Dipstick Biosensor for Detection of Pathogenic *Leptospira*, *Analytica Chimica Acta* (2015), doi: 10.1016/j.aca.2015.11.015.

Development of Multiplex Loop Mediated Isothermal Amplification (m-LAMP) Label-based Gold Nanoparticles Lateral Flow Dipstick Biosensor for Detection of Pathogenic *Leptospira*

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This work has been partially presented at Postgraduate Colloquium on Medical Sciences 2015, on 25-26th May 2015, at UiTM Sg Buloh, Selangor, Malaysia (Abstract in Appendix A)

ABSTRACT

In recent years extensive numbers of molecular diagnostic methods have been developed to meet the need of point-of-care devices. Efforts have been made towards producing rapid, simple and inexpensive DNA tests, especially in the diagnostics field. We report on the development of a label-based lateral flow dipstick for the rapid and simple detection of multiplex loop-mediated isothermal amplification (m-LAMP) amplicons. A label-based m-LAMP lateral flow dipstick assay was developed for the simultaneous detection of target DNA template and a LAMP internal control. This biosensor operates through a label based system, in which probe-hybridization and the additional incubation step are eliminated. We demonstrated this m-LAMP assay by detecting pathogenic *Leptospira*, which causes the re-emerging disease Leptospirosis. The lateral flow dipstick was developed to detect of three targets, the LAMP target amplicon, the LAMP internal control amplicon and a chromatography control. Three lines appeared on the dipstick, indicating positive results for all representative pathogenic *Leptospira* species, whereas two lines appeared, indicating negative results, for other bacterial species. The specificity of this biosensor assay was 100% when it was tested with 13 representative pathogenic *Leptospira* species, 2 intermediate *Leptospira* species, 1 non-pathogenic *Leptospira* species and 28 other bacteria species. This study found that this DNA biosensor was able to detect DNA at concentrations as low as 3.95×10^{-1} genomic equivalent ml^{-1}. An integrated m-LAMP and label-based lateral flow dipstick was successfully developed, promising simple and rapid visual detection in clinical diagnostics and serving as a point-of-care device.

(keywords: Label-based multiplex loop-mediated isothermal amplification; Lateral flow dipstick; Pathogenic *Leptospira*; Point-of-care)

1. Introduction

The existence of numerous nucleic acid analytical techniques has generated a significant increase in their application in many fields, especially in clinical diagnostics. Molecular technology has become a crucial tool for identifying and amplifying genes, to manage the diagnosis, prevention and control of diseases in living beings. Polymerase chain reaction (PCR) is considered to be a robust nucleic acid amplification technique in molecular analysis platforms. Conventional PCR has been modified and developed into various versions such as multiplex PCR, nested PCR, real-time PCR, quantitative PCR, immunocapture PCR and others. However, its limitations such as long processing time, the need for thermocycling equipment and the requirement for expertise to handle the procedure, have spurred the researchers to develop other superior DNA amplification techniques.

To date, various isothermal amplification methods, such as transcription-mediated amplification, nucleic acid sequence-based amplification, signal-mediated amplification and strand displacement amplification have been developed for use in molecular analyses [1]. In particular, Loop-mediated isothermal amplification (LAMP) has been widely applied in molecular diagnostics because it promises of high sensitivity, specificity and speed [2]. Several studies have demonstrated the detection of various pathogens by using the LAMP method [3-6]. The detection of LAMP amplicons has become a major concern because simplifying diagnostic tools is a major concern. Various LAMP detection methods such as turbidimeters [7], fluorescent agents [8-10], colorimetric agents [11-13], lateral flow dipsticks [14-18] and lab-on-a-chip devices [19] have been developed. In particular, lateral flow dipsticks have been employed to achieve point-of-care diagnostics. Currently, rapid, reliable and affordable point-

of-care tests are needed especially by people in developing countries that do not have access to laboratory services. Here we report on a label-based lateral flow dipstick for the detection of multiplex LAMP (m-LAMP) amplicons. In this study, we eliminate the use of label-probe for this biosensor, thus there is no incubation step prior to lateral flow dipstick detection.

We demonstrate the simplicity and feasibility of this biosensor by detecting the *LipL32* gene of pathogenic *Leptospira* which, causes leptospirosis. Leptospirosis is a re-emerging disease with clinical features similar to those of a range of other infectious diseases, including scrub thypus, dengue and malaria [20] . The previous available laboratory diagnostic methods include microscopic demonstration, antigen detection, cultivation, antibody detection and DNA detection [21]. However, these methods do not permit an early definitive diagnosis, lack sensitivity and are quite laborious, requiring highly skilled personnel to perform the test. Many previous studies have applied LAMP for the detection of leptospirosis [10, 22-24]. In this study, we developed a m-LAMP assay to detect *Leptospira* that uses a LAMP internal control. Amplification of the internal control is important to rule-out false-negative results in the amplification assay [25]. Thus we applied an internal control to this integrated m-LAMP and label-based lateral flow dipstick.

2. Experimental Section

2.1. Reagents and apparatus

Anti-mouse polystyrene particles, anti-digoxigenin coated polystyrene particles and streptavidin polystyrene particles were purchased from Spherotech Inc. (IL, USA). Colloidal gold solution

(40 nm) was from Heron Diagnostics (Pathumthani, Thailand). All reagents used in this study were analytical reagent grade and all solutions were prepared by using ultrapure water (> 18 MΩ) from Millipore Milli-*Q* water purification system (Billerica, USA). FUSION5 membranes were from Whatman (Kent, UK), absorbent pads were from a Millipore (MA, USA), and plastic adhesive backing cards were from G&L Precision Die-cutting (Amstelveen, The Netherlands). LAMP primers were synthesized by Integrated DNA Technologies (Science Park RD, Singapore). LAMP reagents, including anhydrous betaine and magnesium sulphate heptahydrate were obtained from Sigma-Aldrich (Steinheim, Germany), WarmStart 2.0 *Bst* DNA polymerase was from New England Biolabs (Ipswich, MA), and the dNTP mixture was obtained from Thermofisher Scientific (MA, USA).

2.2. Leptospira species and control bacterial strains

Leptospira species were obtained from Institute for Medical Research (IMR) and Universiti Putra Malaysia (UPM) and were cultured in EMJH media and incubated at 30°C for one week. Other bacterial species were cultured on LB agar overnight at 37°C. Genomic DNA of cultured *Leptospira* and other bacteria was extracted using NucleoSpin® Tissue from Machenery-Nagel (Düren, Germany), according to the manufacturer's instructions. Genomic DNA was diluted to 20 ng/µl using sterile ultrapure water. The genome size of *Leptospira interrogans* (4.659 MB) was used to calculate the number of genome equivalents in the assay. Concentration of diluted genomic DNA (20 ng/µl) was equivalent to approximately 3.95 x 10^6 genome equivalents ml^{-1}.

2.3. Design of LAMP assay primers

Primers for pathogenic *Leptospira* and the LAMP internal control were designed by using conserved genes of the pathogenic strains. A set of primers was designed by using Primer Explorer V4 software (Eiken Chemical Co., Tokyo, Japan) based on the nucleotide sequence of the *LipL32* gene of *Leptospira interrogans* serovar Hebdomadis (GenBank accession no. AY609328.1). A set of 5 primers including a forward inner primer (FIP), a backward inner primer (BIP), a forward outer primer (F3), a backward outer primer (B3) and a loop primer (LB for pathogenic *Leptospira* and LF for the LAMP internal control) were selected for each of the two genes. All primers were evaluated for their specificity by conducting a BLAST search with sequences in GenBank before use in LAMP assays. The sequences of all primers used for amplification of the *LipL32* gene region and LAMP internal control are shown in **Table 1**.

2.4. M-LAMP assay

M-LAMP assay was carried out in a total of 50 µl reaction mixture containing 1X Isothermal Amplification Buffer, 0.8 M betaine, 6 mM $MgSO_4$, 0.6 mM dNTP mix, 8 U of *Bst* 2.0 WarmStart DNA polymerase, target and internal control primer mixtures (5 µM forward and reverse outer primer, 40 µM forward and reverse inner primer and 20 µM loop primer), target genomic DNA template and internal control plasmid. The optimization was performed using genomic DNA extracted from *Leptospira interrogans* serovar *Hebdomadis*. The reaction mixture was incubated at 63°C for 1 hour, followed by termination step at 80°C for 10 min. Amplified products were analysed using 2% agarose gels containing RedSafe staining solution, followed by DNA visualization using a UV transilluminator.

2.5. Construction of the lateral flow dipstick

2.5.1. Functionalization of gold nanoparticles

Conjugation of colloidal gold nanoparticles and mouse anti-fluorescein antibody was performed as described by Ang, Yu and Yean [26]. Briefly, a total of 10 ml of 40 nm colloidal gold nanoparticles were adjusted to pH 8.0. Then, 22 µg of anti-FITC antibody from Pierce was added followed by gentle shaking at room temperature for 1 hour. Bovine serum albumin (BSA) from Amresco (Solon, USA) with concentration 1% (w/v) was added and allowed to mix for another 1 hour. Colloidal gold-anti-FITC antibody conjugate was centrifuged at 10, 000 *g* for 30 min at 4 °C. Finally, the pellet was suspended in 10 mM phosphate buffer (PB) containing 1% (w/v) BSA and 0.05% (w/v) sodium azide.

2.5.2. Preparation of the lateral flow dipstick

The lateral flow dipstick was designed to detect three targets: a chromatography control, a LAMP internal control template, and the target DNA template. Construction of this biosensor was based on Chua, Yean, Ravichandran, Lim and Lalitha [27]. Two compartments, including a reaction pad and an absorbent pad were assembled to form the dipstick by using FUSION 5 membrane and a cellulose fibre sample pad, respectively. The reaction pad was fixed on the backing card, and the absorbent pad was assembled on the backing card by overlaying the reaction pad by 2 mm. Capture reagents, which include anti-mouse polystyrene particles, anti-digoxigenin coated polystyrene particles and streptavidin polystyrene particles were affixed on

the first line as the chromatography control line, the second line as the LAMP internal control line and the third line as the target test line, respectively at a rate of 0.4 µl/cm. The membrane was dried in dry cabinet for at least 2 hours prior to use. The dipsticks were generally cut into 5-mm strips using a Matrix 2360 Programmable Shear (Kinematic Automation, Twain Harte, Calif.). The dipsticks were stored in a dry cabinet immediately for future use.

2.5.3. Lateral flow dipstick assay

LAMP-buffer mixture was prepared by mixing LAMP amplicon and running buffer [10 mM PBS (pH 7.4), 1.0 %(v/v) Tween-20], and applied on the lateral flow dipstick. Then, the lateral flow dipstick was immersed into 20 µl of buffer, followed by 20 µl of gold nanoparticles coated with mouse anti-FITC antibody (OD_{525} of 4), the lateral flow dipstick was then placed into contact with another 100 µl of running buffer. LAMP amplicon detection was indicated by the presence of red lines on the reaction pad.

2.6. Sensitivity of m-LAMP by gel electrophoresis and lateral flow dipstick

The limit of detection of the m-LAMP assay was determined by conducting a 10-fold serial dilutions of pathogenic *Leptospira* DNA copies. The amplified DNA was detected by 2% agarose gel electrophoresis with RedSafe staining solution and by lateral flow dipstick. The sensitivity test was compared with multiplex PCR.

2.7. Multiplex PCR for sensitivity comparison

Multiplex PCR was conducted to compare the sensitivity of the developed m-LAMP assay. A total volume of 20 µl reaction mixture containing 1X buffer, 2.5 mM $MgCl_2$, 0.16 mM dNTPs mix, 1 µM of each forward and reverse outer primers for both target *Leptospira* and internal control, and 0.75 U of *Taq* DNA polymerase. PCR was performed in a thermal cycler for 30 cycles. Each cycle consisted of initial denaturation step at 95 °C for 3 min, followed by denaturation at 94 °C for 30 s, annealing 58 °C for 30 s, and extension at 72 °C for 1 min, followed by an extra annealing step of 58 °C for 30 s and a final extension step at 72 °C for 5 min. The amplification products were analyzed using 1.5 % agarose gel electrophoresis, stained with RedSafe, and visualized under UV illumination.

2.8. Specificity of m-LAMP by gel electrophoresis and lateral flow dipstick

The specificity of the LAMP primers was evaluated using extracted genomic DNA from 13 other pathogenic *Leptospira* serovars, 2 intermediate *Leptospira* species, 1 nonpathogenic *Leptospira* species, and 27 other bacterial species. Internal control plasmid DNA (200 pg/µl) was used as the LAMP internal control. The m-LAMP products were analysed by using 2% agarose gel electrophoresis and the lateral flow dipstick.

3. Result and discussions

3.1. Design of m-LAMP primers

Primers in the m-LAMP were designed to targets, pathogenic *Leptospira* and the LAMP internal control. The primers were designed based on highly conserved regions for both targets. Primers targeting *LipL32* gene was selected because it is claimed to be highly conserved region among pathogenic *Leptospira* species [28]. The primer sets were screened for their specificity by conducting BLAST searches, and the results showed that the primers were highly specific to their targets. The primer sets were also checked for self-dimerization and hetero-dimer formation by using OligoAnalyzer. Optimization on loop primer was done and result showed that the inclusion of loop primer for both targets accelerates the LAMP assay (data not shown). In addition, optimization of this multiplex assay showed that there is no primers' cross-reactivity. There were no amplified products when the internal control plasmid was inserted into a LAMP assay that contained a single set of target pathogenic *Leptospira* primers (**Fig. 1**, lane 2). Pathogenic *Leptospira* DNA template was also not amplified when it was inserted into a LAMP assay that contained a single set of internal control primers (**Fig. 1**, lane 4). A comparison of species-specific ladder-like patterns of m-LAMP products is shown in **Fig. 1** (lanes 5 to 7). Primers used in this m-LAMP assay were labelled with a biotin tag at the 5' end of the backward loop primer for pathogenic *Leptospira*, and a digoxigenin tag at the 5' end of the forward loop primer of the LAMP internal control. A fluorescein tag was used to label at the 5' end of the backward inner primer and the forward inner primer of pathogenic *Leptospira* and the LAMP internal control, respectively. This is because a backward loop primer for pathogenic *Leptospira* and a forward loop primer for the LAMP internal control were used in this multiplex assay.

3.2. M-LAMP reaction conditions

Genomic DNA of *Leptospira interrogans* serovar Hebdomadis was used for the assay optimization. Optimization of the m-LAMP assay was performed for the concentration of each reagents, incubation time and incubation temperature. The optimal m-LAMP assay conditions showed the clearest characteristic ladder-like pattern characteristic on 2% agarose gel electrophoresis. The results showed that the optimal m-LAMP assay was obtained with 0.8 M betaine, 6 mM $MgSO_4$ and 0.6 mM dNTP mix (data shown in Supplementary data). The optimal ratio of outer to inner primer was found to be 1:8, because the amplicons showed the clearest characteristic ladder-like pattern. The reaction mixture was incubated separately at 60 °C, 63 °C and 65 °C, for 60 min and subsequently terminated at 80 °C for 10 min. Based on the result, at 63 °C, the m-LAMP amplicons showed the clearest pattern (data shown in Supplementary data) and thus 63 °C was considered to be optimal temperature for the m-LAMP assay. The reaction mixtures were also incubated separately for 15 min, 30 min, 45 min, or 60 min. The m-LAMP amplicons could be observed as early as 30 min. However, 60 min was selected as the optimal incubation time because the amplicons showed the strongest signals. Moreover, 60 min was reported as the optimal incubation time to ensure the detection of target template at lower concentration.

3.3. Detection of LAMP amplicons

The schematic illustration in **Fig. 2** shows the principle of the DNA biosensor. Each line captures its specific recognition of the hapten labels on the LAMP amplicons. The amplicons from pathogenic *Leptospira* were biotin- and fluorescein-labelled, as derived from hapten-labelled primers, which are a fluorescein-labelled backward inner primer and a biotin-labelled backward loop primer. The amplicons from the LAMP internal control were digoxigenin- and fluorescein-labelled, as generated from a fluorescein-labelled forward inner primer and a biotin-labelled forward loop primer. The biotin/fluorescein-labelled target amplicons are captured by the streptavidin affixed on the first line, known as the test line. The LAMP internal control amplicons, which are digoxigenin/fluorescein-labelled, are captured by the anti-digoxigenin antibody affixed on the second line, the LAMP internal control line. The anti-fluorescein antibodies conjugated with gold nanoparticles are captured by fluorescein-labelled amplicons. Interpretation of a positive result is based on the presence of three red lines on the reaction pad. However, the appearance of a red line on the LAMP internal control line is not necessary for the interpretation of a positive biosensor result. This is due to inhibitory effects caused by the presence of very large amounts of target DNA template. However, a red line must appear on the LAMP internal control line to validate a true negative result. The role of the third line, which is known as the chromatography control line, is to assess the functionality of each individual test strip. In all test results, a red line must appear on the chromatography control line, which proves that the chromatography flow is working well. Excess anti-fluorescein antibodies conjugated with gold nanoparticles are captured on that line, which is affixed with goat anti-mouse polystyrene particles and validates the working condition of the biosensor.

3.4. Construction of the lateral flow dipstick

An adequate amount of gold conjugates available for binding to the labelled amplicons produces a biosensor with good performance, because it influences the intensity of the lines. However, excess gold conjugate is not an optimal condition for a biosensor because it may prolong the assay time due to extended release time through the pad [26]. In this study, the amount of gold conjugate was selected at an OD_{525} of 4 for the optimal signal intensity. LAMP amplicons mixed with running buffer that was supplemented with different concentrations of Tween-20 were tested. Tween-20 is commonly used in lateral flow dipsticks because this additive promotes the release of gold conjugates and avoids nonspecific lines on the reaction pad [29, 30]. It was found that increasing the Tween-20 concentration increased the intensity of the red line, thus 1.0% Tween-20 is the optimal concentration to be used in running buffer.

3.5. Specificity of the m-LAMP assay by using gel electrophoresis and the lateral flow dipstick

A specificity test was conducted using extracted genomic DNA from pathogenic *Leptospira* serovars, intermediate *Leptospira* species, nonpathogenic *Leptospira* species, and other bacterial species. The data in **Table 2** reveal that no cross-reactions were obtained in m-LAMP-gel electrophoresis.

3.6. Sensitivity of m-LAMP assay by lateral flow dipstick detection

The detection of pathogenic *Leptospira* by m-LAMP-lateral flow dipstick reached a limit at the 3.95×10^{-1} genomic equivalent per reaction [**Fig. 3 (A)**]. The gene, Lipl32, detected in this

study is located as a single copy on chromosome I of L. interrogans [31]. Hence the limit of detection is equal to 3.95 x 10^{-1} cell. This result was compared with 2% agarose gel analysis [**Fig. 3 (B)**]. It was observed that clear visible red lines were observed for both the test line and the LAMP internal control line. Agarose gel electrophoresis results showed limit detection at 3.95 x 10^{0} genomic equivalents per reaction. Based on the result from multiplex PCR, it showed the limit of detection at 3.95 x 10^{4} genomic equivalents per reaction [**Fig. 3 (C)**]. This result suggests that the developed biosensor can provide the sensitivity better than agarose gel detection and PCR method. In addition, it is also applicable on the real sample diagnosis because it reached the estimated density of *Leptospira* in blood sample, which is 8.0 x 10^{1} – 3.9 x 10^{4} leptospires/ml in blood [32].

4. Conclusions

The developed label-based lateral flow dipstick biosensor avoids the use of labelled capturing probes, allowing the probe design step to be eliminated. The detection of LAMP amplicons through probe-amplicon hybridization may cause nonspecific detection if the probe is not properly designed. Thus, we simplified the detection platform by introducing this label-based lateral flow dipstick biosensor. Specificity of the test platform can be achieved through appropriate design of the LAMP primer sets. Moreover, the end-user can skip the incubation step prior to detection, which reduces the detection time. Although the cost of the detection assay is slightly higher than that of PCR-gel electrophoresis, this detection platform promises to be a highly sensitive, specific, safe and rapid detection system. In addition, this user-friendly biosensor eliminates the need for skilled personnel and users can obtain the rapid visual test

results in minutes. Due to these advantages, this m-LAMP-lateral flow dipstick biosensor could be applicable for use in point-of-care settings.

Acknowledgements

This work was funded by Prototype Development Research Grant Scheme (203/PPSP/6740018), Research University Grant (1001/PPSP/812144) and ScienceFund Grant (305/PPSP/6113220). The first author is financially supported by Ministry of Education Malaysia through MyBrain15 Fellowship scheme. We thank to Faculty of Veterinary Medicine, UPM and IMR Malaysia for *Leptospira* strains. We also thank the Institute for Research in Molecular Medicine, USM for the cooperation and facilities provided.

LIST OF TABLES

LIST OF FIGURES

is affixed with streptavidin polystyrene particles. The LAMP internal control is digoxigenin- and fluorescein-labelled and is captured on the LAMP internal control line, which is affixed with monoclonal anti-digoxigenin coated polystyrene particles. Anti-fluorescein monoclonal antibody conjugated to gold nanoparticles accumulates and thus produces a visual signal. The chromatography control line validates the working condition of the biosensor, in which excess anti-fluorescein antibody conjugated to gold nanoparticles is captured by goat anti-mouse polystyrene particles affixed on that line. B, biotin; D, digoxigenin; F, fluorescein; G, anti-fluorescein antibody conjugated with gold nanoparticles.

Fig. 3 Comparison of analytical sensitivity results by using two different LAMP detection methods and multiplex PCR. (A) 2% gel electrophoresis detection. (B) label-based lateral flow dipstick detection. (C) Multiplex PCR. M, 100 bp PLUS DNA ladder; N, Negative control; 1 – 2, 10-fold serial dilution of 3.95×10^6 genome equivalents ml^{-1}.

LIST OF APPENDIX

Appendix A	Abstract for Postgraduate Colloquium on Medical Sciences 2015, on 25-26th May 2015, at UiTM Sg Buloh, Selangor, Malaysia
Appendix B	Supplementary data

Table 1. Oligonucleotide sequence of primers for m-LAMP assay

Target	Oligonucleotide	Sequence (5' →3')	Length (mer)
Pathogenic *Leptospira*	F3	GTAAGCGACGCTTTCAAAG	19
	FIP	CCGACATTCTTTCTACACGGATC GAATTCCCCCAGAAGAAAAATC AATGC	50
	B3	TGTCTCTCTTCTTTATAAGTATCG	24
	BIP	6-FAM- TTATGCCTGACCAAATCGCCGAA TTCTCACCATCATCATCATCGT	45
	LB	Bio- CTGCGAAAGCAAAACCAGTTC	21
LAMP internal control	F3	TCAGCGACAATCGTTCAACT	20
	FIP	6-FAM- CGACCTGTAAGTTCAGCACGGTT TTTTTCAATGGCAAAAAAGCCCA C	47
	B3	TCAAGCTGTTCAACGGGAAT	20
	BIP	GTGCGCGGGTCGAAACTTATGAG AATTCAATTGCGGATCAGGCTTT GT	48
	LF	DigN- CCAAGAAAATTGCTTAAACGCA GTG	25

6-FAM, carboxyfluorescein; Bio, biotin; DigN, digoxigenin

Table 2. Analytical specificity results of detection of m-LAMP on lateral flow dipstick

Bacterial strains	M-LAMP	
	Gel electrophoresis detection	Lateral flow dipstick detection
Pathogenic *Leptospira* serovar		
Canicola	+	+
Pomona	+	+
Australis	+	+
Bataviae	+	+
Icterohaemorrhagiae RGA	+	+
Hebdomadis	+	+
Celledoni	+	+
Ballum	+	+
Autumnalis	+	+
Pyrogenes	+	+
Tarassovi	+	+
Copenhageni	+	+
Javanica	+	+
Intermediate *Leptospira* species		
Leptospira liceraise serovar Varillal	-	-
Leptospira fainei serovar Hurtsbridge	-	-
Nonpathogenic *Leptospira* species		
Leptospira biflexa serovar Patoc	-	-
Other bacteria species		
Bacillus subtilis	-	-
Enterococcus faecium	-	-
Enterococcus faecalis	-	-
Enterococcus gallinarum	-	-
Listeria monocytes	-	-
Staphylococcus aureus	-	-
Staphylococcus epidermis	-	-
Group A *Streptococcus*	-	-
Group B *Streptococcus*	-	-
Group F *Streptococcus*	-	-
Group G *Streptococcus*	-	-
Vibrio cholera	-	-
Vibrio cincinnatiensis	-	-
Vibrio fluvialis	-	-

Vibrio mimicus	-	-
Vibrio parahaemolyticus	-	-
Vibrio vulnificus	-	-
Salmonella Typhi	-	-
Shigella flexneri	-	-
Enteroinvasive *Escherichia coli*	-	-
Enterohaemorrhagic *Escherichia coli*	-	-
Enteropathigenic *Escherichia coli*	-	-
Enterotoxigenic *Escherichia coli*	-	-
Yersenia enterocolitica	-	-
Klebsiella pneumonia	-	-
Aeromonas hydrophila	-	-
Actinobacter baumanii	-	-

+, Positive result; -, Negative result

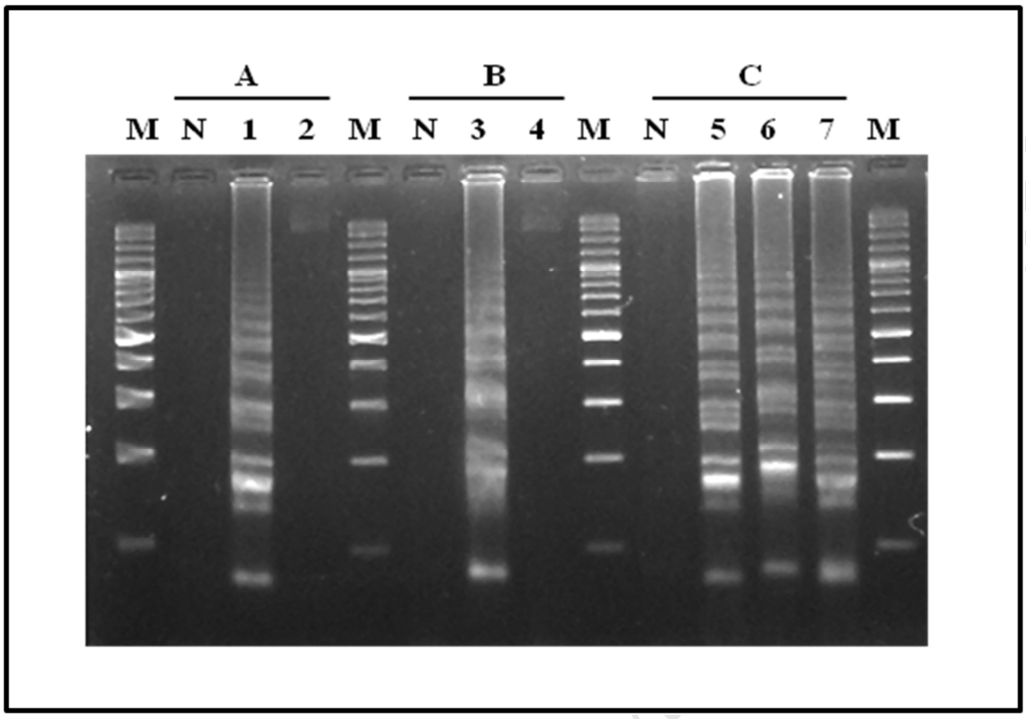

Fig. 1 Primer cross-reactivity assesment and comparison of species-specific ladder patterns of multiplex LAMP products. M, 100 bp PLUS DNA ladder; N, Blank; A, LAMP assay with single set of target pathogenic *Leptospira* primers; B, LAMP assay with a single set of internal control primers; C, LAMP assay with 2 sets of primers targeting pathogenic *Leptospira* and the internal control; Lanes 1, 4 and 5, pathogenic *Leptospira* DNA template; Lanes 2, 3 and 6, internal control plasmid; lane 7, both pathogenic *Leptospira* DNA template and internal control plasmid.

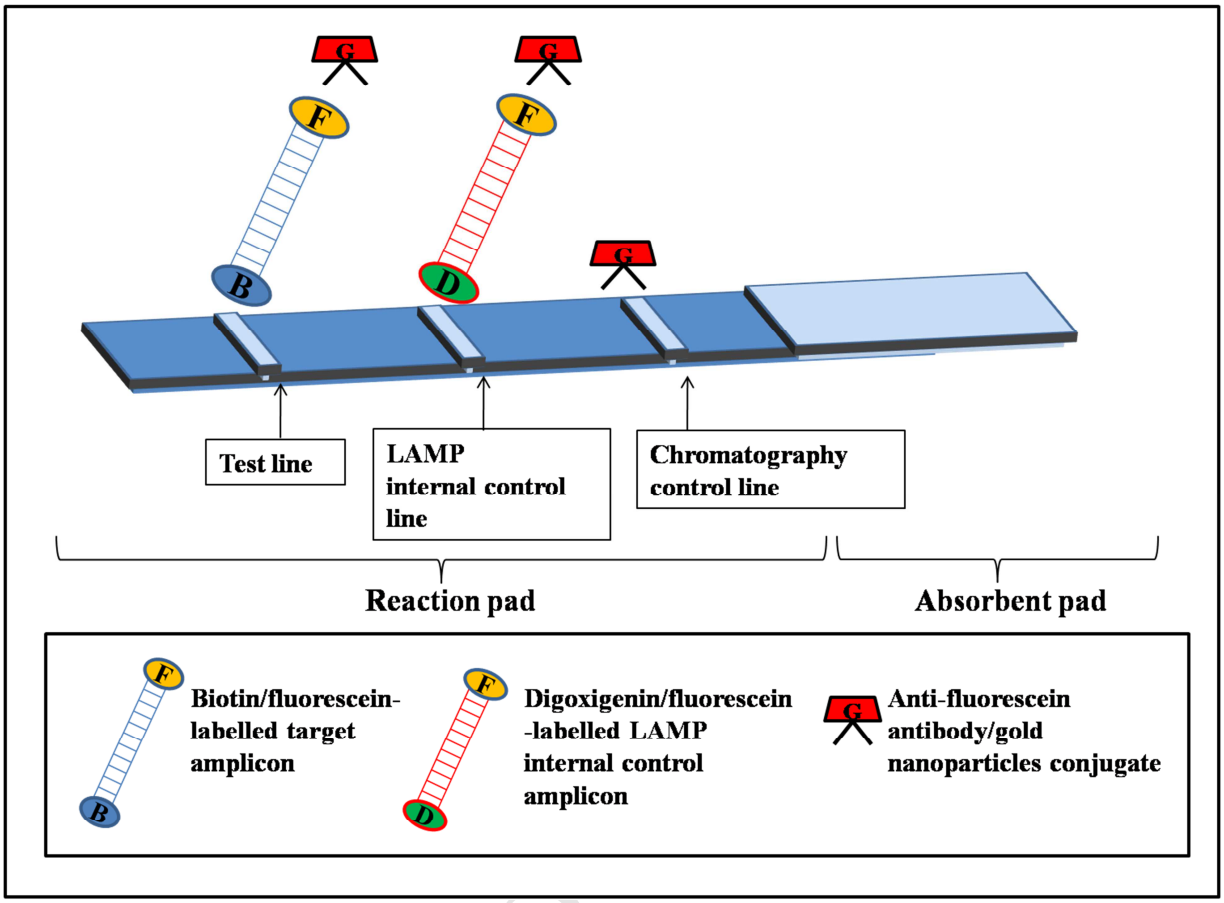

Fig. 2 Schematic illustration of the principle underlying the visualization of LAMP amplicons using a lateral flow dipstick. The lateral flow dipstick is composed of an absorbent pad and a reaction pad. The target organism is biotin- and fluorescein-labelled and is captured on the test line, which is affixed with streptavidin polystyrene particles. The LAMP internal control is digoxigenin- and fluorescein-labelled and is captured on the LAMP internal control line, which is affixed with monoclonal anti-digoxigenin coated polystyrene particles. Anti-fluorescein monoclonal antibody conjugated to gold nanoparticles accumulates and thus produces a visual signal. The chromatography control line validates the working condition of the biosensor, in which excess anti-fluorescein antibody conjugated to gold nanoparticles is captured by goat anti-mouse polystyrene

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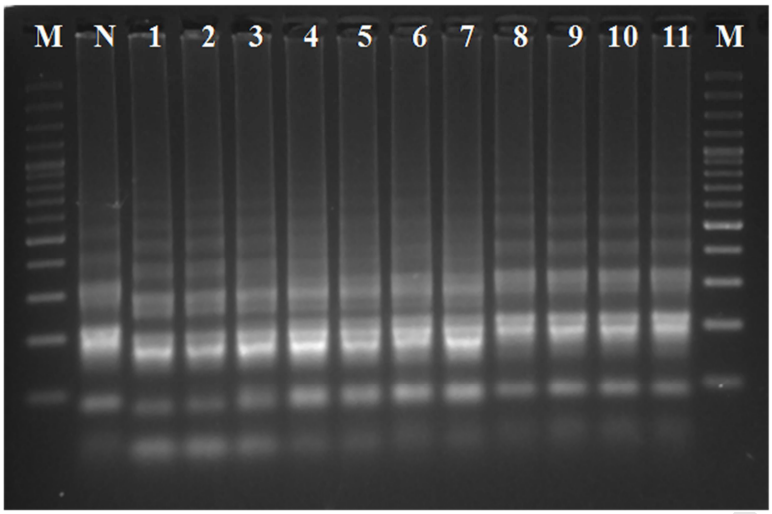
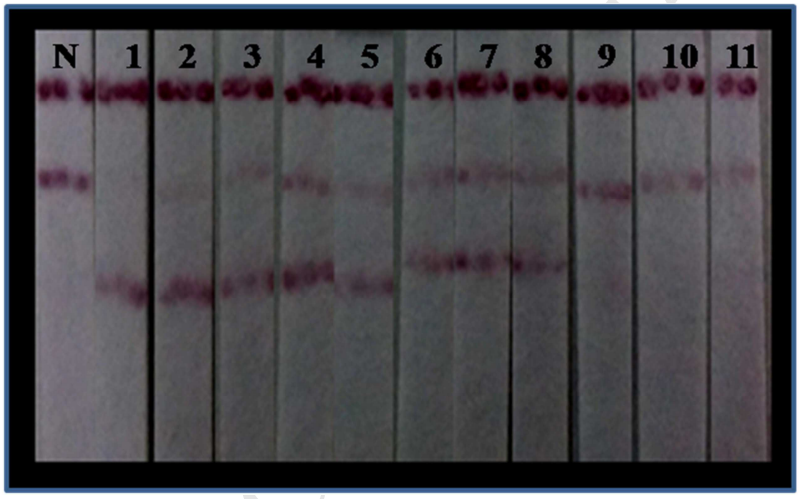
(A)



(B)



(C)

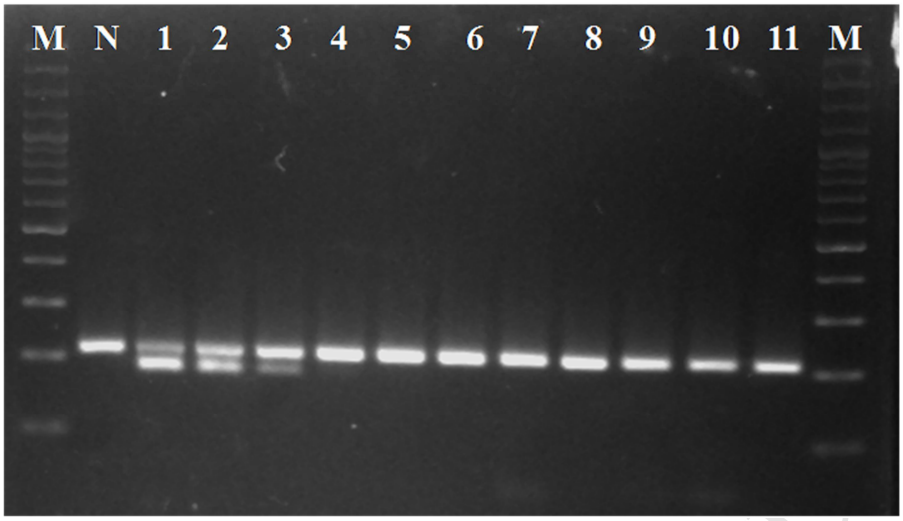


Fig. 3Comparison of analytical sensitivity results by using two different LAMP detection methods and multiplex PCR. (A) 2% gel electrophoresis detection. (B) label-based lateral flow dipstick detection. (C) Multiplex PCR. M, 100 bp PLUS DNA ladder; N, Negative control; 1 – 2, 10-fold serial dilution of 3.95 x 10^6 genome equivalents ml^{-1}.

Appendix A

Abstract for Postgraduate Colloquium on Medical Sciences 2015, on 25-26th May 2015, at

UiTM Sg Buloh, Selangor, Malaysia

DEVELOPMENT OF INTEGRATED MULTI-LOOP MEDIATED ISOTHERMAL AMPLIFICATION (multi-LAMP) AND LABEL-BASED LATERAL FLOW DIPSTICK FOR DETECTION OF PATHOGENIC *Leptospira* sp.

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INTRODUCTION: *Leptospira* is a causative agent of an emerging disease, Leptospirosis. This disease causes misleading diagnostic since its clinical symptoms are being confused with other diseases. Hence, rapid and specific detection of the presence of this causative agent is very crucial for the need of proper and rapid treatment. This study was aimed to develop a specific multi-LAMP assay for the detection of pathogenic Leptospira with the presence of internal control and coupled with lateral flow dipstick as a point-of-care (POC) device.

METHODS: A total volume of 25 µL LAMP reagents mixture, including 1X buffer, 0.8 M betaine, 6 mM MgSO4, 0.6 mM dNTPs mix, 8U of *Bst* 2.0 Warmstart DNA polymerase, target and internal control primers mixture (5 pmol/ µL of each forward and reverse outer primer, 40 pmol/ µL of each forward and reverse inner primer and 20 pmol/ µL of loop primer), target DNA template and internal control template was prepared in a tube. The reagents mixture was then incubated at 63 °C for 1 hour, followed by termination at 80 °C for 10 min. LAMP amplicons detection was done on lateral flow dipstick. Three lines appeared on the dipstick indicate positive result for pathogenic Leptospira, while two lines indicate negative result.

RESULTS & DISCUSSION: The developed integrated multi-LAMP assay gave positive results for all 15 representatives pathogenic *Leptospira*, while negative results for 2 intermediate *Leptospira*, 1 non-pathogenic *Leptospira* and 10 other bacteria species.

CONCLUSION: An integrated multi-loop mediated isothermal amplification and label-based lateral flow dipstick was successfully developed for the detection of pathogenic *Leptospira* as a point-of-care device.

KEY WORDS: Loop-mediated isothermal amplification (LAMP), lateral flow dipstick, leptospirosis

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Highlights:

- We develop the fool-proof (internal control included) multiplex Loop Mediated Isothermal Amplification (m-LAMP) label-based lateral flow dipstick biosensor for detection of pathogenic *Leptospira*
- We design primers for multiplex LAMP that targets the conserved *LipL32* gene of pathogenic *Leptospira* and LAMP internal control.
- Inner primers for both pathogenic *Leptospira* and LAMP internal control are labeled with either biotin or digoxigenin tag, while loop primers are labeled with fluorescein tag.
- Gold nanopartices is coupled with anti-fluorescein antibody, to give visualize red line on the lateral flow dipstick.
- Positive results show three red lines while negative results show two red lines on the lateral flow dipstick.