Detection of Pathogenic Leptospires by Loop-Mediated Isothermal Amplification Targeting *LipL32* Gene

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Background: *Leptospirosis is a worldwide re-emerging infectious disease caused by pathogenic leptospires including Leptospira interrogans.*
Objective: *In the present study, a loop-mediated isothermal amplification (LAMP) was developed to detect L. interrogans using lipL32 as a gene target.*
Material and Method: *Four specific primers were designed based on the conserved region of lipL32 gene of various serovars of pathogenic leptospires. LAMP reaction was performed at 65°C for 1 hour. The LAMP products were detected by agarose gel electrophoresis and fluorescence dye.*
Results: *The lipL32 LAMP assay showed highly specificity to the reference stains of L. interrogans serovar Autumnalis, Bataviae, Javanica, Pyrogenes, Icterohaemorrhagiae, and Saigon. No product was produced from non-pathogenic leptospire (L. biflexa), human, or Escherichia coli. The lower limit of detection analyzed by agarose gel electrophoresis and fluorescence dye visualization was 0.02 pg/µl which equivalent to 4 genomic equivalents/reaction. Moreover, the clinical strain of leptospires including pathogenic and intermediate group of L. interrogans were detected by lipL32 LAMP.*
Conclusion: *The developed lipL32 LAMP is high specificity and sensitivity that can be applied to detect pathogenic leptospires in clinical samples.*

Keywords: *Loop-mediated isothermal amplification, LAMP, Leptospira interrogans, LipL32, Detection, Leptospirosis*

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Leptospirosis is an important zoonotic infectious disease causing public health concerns in developing countries[1]. The causative agent of leptospirosis is pathogenic leptospira that has more than 300 serovar[2,3]. Clinical signs of leptospirosis are non-specific and similar to symptoms of other infectious diseases that arise in the same regions, including dengue fever, scrub typhus, and malaria[4,5]. The laboratory diagnosis of leptospirosis is essential to provide an accurate diagnosis and effective patient management. Currently, the microscopic agglutination test (MAT) is the most commonly used for serologic diagnostic gold standard[5], but it is ineffective for early diagnosis[6,7]. Other techniques for diagnosis such as enzyme-linked immunosorbent assay and dark-field microscopy have low sensitivity and are time consuming[1,8]. In addition, the molecular analysis such as polymerase chain reaction (PCR) and real-time PCR, which are rapid, specific, and sensitive methods were developed for diagnosis of leptospirosis. However, the PCR instruments are expensive and unsuitable for routine use in many laboratories of developing countries[9,10].

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method, which amplifies a target DNA under isothermal conditions with high specificity and sensitivity. The products of LAMP are stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops due to annealing between alternately inverted repeats of the target within the same strand[11]. The result of LAMP reaction can be followed by using agarose gel electrophoresis, or a fluorescent

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dye such as calcein to measure the reaction turbidity with naked eye[12]. The LAMP method has been developed for molecular detections of pathogenic bacteria and viruses including 16S rRNA (*rrs* LAMP)[5,13,14] and *lipL41* gene (*lipL*41 LAMP) of pathogenic leptospires. The lower limit of detection was described to be approximately 10 genomic equivalents (GEs)/reaction for 16S rRNA[5] and 100 GEs/reaction for *lipL41* gene[8]. The *rrs* LAMP exhibited low specificity, therefore, it was not recommended for diagnosis of leptospire in clinical sample[5].

The *lipL32* gene of pathogenic leptospira encodes an outer membrane lipoprotein. It is purposed to be a virulence factor that does not appear in non-pathogenic leptospire[15-17]. Previously, the real-time PCR developed for leptospiral *lipL32* gene detection was shown to be specific to pathogenic leptospires and most sensitive compare to *secY* and *lfb1* genes[18]. Real-time PCR assay using a TaqMan probe targeting *lipL32* detected 20 GEs/reaction with a 95% cutoff value[19]. The gene *lipL32* was used as the amplification target for recombinase polymerase amplification assay (RPA)[20]. In this study, loop-mediated isothermal amplification was developed to simply detect *lipL32* gene of pathogenic leptospires.

Material and Method

Bacterial strains and growth condition

Six reference strains of pathogenic leptopsira including *L. interrogans* serovar Bataviae (serogroup Bataviae), Autumnalis (serogroup Autumnalis), Saigon (serogroup Louisiana), Icterohaemorrhagiae (serogroup Icterohaemorrhagiae), Pyrogenes (Pyrogenes) and Javanica (serogroup Javanica) and one non-pathogenic *L. biflexa* serovar Patoc (serogroup Semaranga) were obtained from the Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand. Ten clinical strains of pathogenic and intermediate group of *Leptopsira* spp. were obtained from Mahidol-Oxford Tropical Medicine Research Unit (MORU), Faculty of Tropical Medicine, Mahidol University, Thailand. The leptospires were grown in Ellinghausen, McCullough, Johnson, and Harris (EMJH) medium (Difco™, USA) at 30°C for 7 days.

Primer design

A set of four primers were designed from the conserved region of *LipL32* outer membrane protein (*lipL32*) gene sequences of *L. interrogans* serovar Copenhageni, Lai and Icterohaemorrhagiae (GenBank accession number GQ204279.1, AY568679.1 and AY423075.1, respectively) using the Primer Explorer V4 software (http://primerexplorer.jp/e/). The sequences of primers used for LAMP assay are listed in Table 1.

Genomic DNA extraction

Leptospires were cultured in 5 ml of EMJH medium at 30°C for 7 days. The cells were collected by centrifugation at 13,000 rpm for 10 min, lysed and deproteinized in 200 µl lysis buffer containing 20 µl of proteinase K (10 mg/ml) at 65°C for 4-6 h. The digested proteins were extracted by using phenol: chloroform (1:1) for 2-3 times. Then, 0.1 volume of 3 M NaOAc (pH 4.8) was added, followed by 0.9 volume of cold 95% ethanol, and incubated at room temperature for 10 min. The DNA solutions were centrifuged at 13,000 rpm for 10 min. The DNA pellet was washed 2-3 times with 70% ethanol, dissolved in sterile-distilled water and stored at -20°C.

Loop-mediated isothermal amplification reaction

LAMP assay was carried out in a total 25 µl reaction mixture containing 2 µM (each) of *lipL32* FIP and BIP primers, 0.2 µM (each) of *lipL32*F3 and B3 primers, 1.4 mM dNTP, 6 mM $MgSO_4$, 1x thermo buffer, 8 U of *Bst* DNA polymerase (New England Biolabs), 0.6 M of betaine (Sigma, St. Louis, MO, USA), and 25 ng of target DNA. The mixture was incubated at 65°C for 60 min. LAMP products were analyzed on a 2% agarose gel electrophoresis or visualized fluorescence dye (Syto 9) under UV light.

Polymerase chain reaction

The PCR assay was performed in 25 µl reaction

Table 1. Nucleotide sequences and length of *lipL32* primers for LAMP assay

Primer	Sequence (5'-3')	Length (bp)
*LipL32*F3	CCA-GGG-ACA-AAC-GAA-ACC-G	19
*LipL32*B3	GCT-TAC-TAA-GTC-TCC-GTC-GC	20
*LipL32*FIP	TAA-ACC-GTC-CGG-CGC-TTG-TCT-TTT-ACT-TCC-CTA-CGG-ATC-TGT-GA	44
*LipL32*BIP	TGG-ATT-CCT-GCC-GTA-ATC-GCT-GTT-TTA-CCG-ATT-TCG-CCT-GTT-GG	44

mixture containing 0.2 µM (each) of *lipL32* F3 and B3 primers, 1x PCR buffer, 1.4 mM dNTP, 1 mM $MgCl_2$ and 2 U of *Taq* DNA polymerase. PCR reaction was carried out for 30 cycles: pre-denaturing at 95°C for 5 min, denaturation at 95°C for 10 sec, annealing at 65°C for 15 sec and extension at 72°C for 30 sec. The PCR products were analyzed on a 2% agarose gel electrophoresis.

Specificity and sensitivity of LAMP

Specificity of LAMP was determined by using DNAs of non-pathogenic leptospire (*L. biflexa* serovar Patoc), *Escherichia coli*, *Streptomyces* spp. SWU10, *Mycobacterium tuberculosis* and human. Sensitivity was determined by using 10-fold serially diluted genomic DNA of *L. interrogans* serovar Autumnalis at a concentration 2,000 pg/µl to 0.0002 pg/µl per reaction.

Detection of clinical strain of pathogenic Leptospira spp.

Ten clinical strains of *Leptospira* spp. including pathogenic and intermediate groups were cultured in 7 ml EMJH medium and their DNAs were extracted by using phenol:chloroform extraction. The purified DNAs were subjected to *lipL32* LAMP as described above.

Results

Specificity of lipL32 LAMP

The analysis of LAMP product by agarose gel electrophoresis (Fig. 1A) showed that genomic DNAs of *L. interrogans* serovar Autumnalis, Bataviae, Pyrogenes, Javanica, Icterohaemorrhagiae and Saigon were amplified by the primers whereas DNAs from *L. biflexa* serovar Patoc and *E. coli* gave no amplified product. The visualization of LAMP product by naked eye revealed that fluorescence dye (Syto9) were observed in the tube containing pathogenic leptospiral DNA but absence in non-pathogenic leptospire (*L. biflexa* serovar Patoc), and no-template DNA (Fig. 1B). These results demonstrated that the *lipL32* LAMP was highly specific for the pathogenic *Leptospira* spp.

Sensitivity of lipL32 LAMP

Sensitivity of *lipL32* LAMP detection was performed by 10-fold serial dilution of genomic DNA of *L. interrogans* serovar Autumnalis at a concentration ranged from 2,000 pg/µl to 0.0002 pg/µl. Agarose gel electrophoretic and fluorescence dye analysis of LAMP product showed that the lower limit of detection was 0.02 pg/µl which equivalent to 4 GE/reaction (Fig. 2).

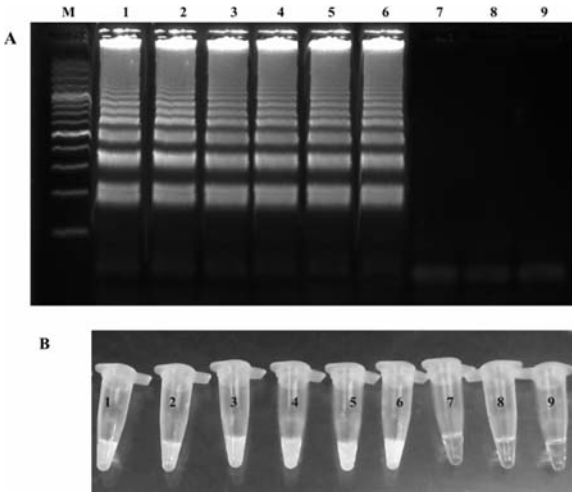

Fig. 1 Specificity of *lipL32* LAMP for detection pathogenic leptospires. (A) Ethidium bromide stained agarose gel electrophoresis of LAMP products of *L. interrogans*; serovar Autumnalis, Bataviae, Pyrogenes, Javanica, Icterohaemorrhagiae and Saigon, respectively (lanes 1-6). Lane 7 to 9, *L. biflexa* serovar Patoc, *E. coli* and negative control. Lane M, 100 bp ladder DNA marker. (B) Visual endpoint detection of LAMP product. Tube 1 to 6, LAMP products of *L. interrogans* serovar Autumnalis, Bataviae, Pyrogenes, Javanica, Icterohaemorrhagiae and Saigon, respectively. Tube 7 to 9, *L. biflexa* serovar Patoc, *E. coli* and negative control, respectively.

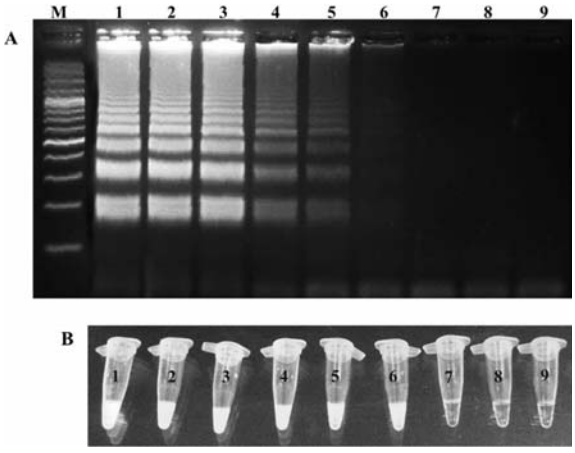

Fig. 2 Sensitivity of *lipL32* LAMP for detection of pathogenic leptospire by agarose gel electrophoresis (A) and fluorescence visualization (B). Lane M, 100 bp ladder DNA marker; lanes/tubes 1 to 8, 10-fold serially diluted DNA *of L. interrogans* serovar Autumnalis from a concentration of 2,000 pg/µl to 0.0002 pg/µl; lane/tube 9, negative control.

Detection clinical strains of pathogenic leptospires

Ten laboratory-proven clinical strains of pathogenic and intermediate group of *L. interrogans* detected by *lipL32* LAMP (Fig. 3A) concordance with fluorescence dye detection (Fig. 3B) and PCR amplification (Fig. 3C). Notably, the difference of intensity of bands or signals were observed (lane 3 and 6). It may be due to the difference in the concentration of the pathogenic strain in the clinical samples. Likewise, BLAST analysis of LAMP primers with *lipL32* gene sequence revealed that at least 29 serovars of pathogenic *Leptospira* spp. showed a 100% sequence homology with all *lipL32* primers (Table 2).

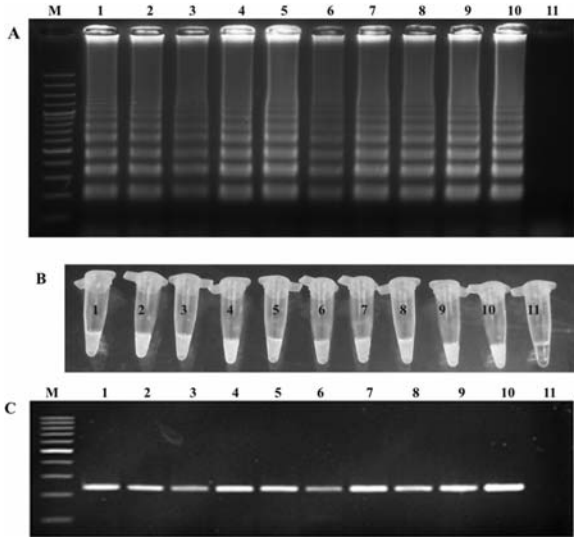

Fig. 3 *LipL32* LAMP assay of clinical isolates of pathogenic leptospire comparing to fluorescence visualization and PCR detection. (A) Agarose gel electrophoresis analysis of LAMP products; lane M, 100 bp ladder DNA marker; lanes 1 to 10, clinical isolate of leptospires, lane 11, negative control. (B) Fluorescence visualization of LAMP products; tubes 1 to 10, clinical isolate of leptospires; tube 11, negative control. (C) Agarose gel electrophoresis analysis of PCR products of clinical isolate of leptospires (lanes 1 to 10); lane 11, negative control.

Discussion

At present, there are a number of molecular techniques used for detection of pathogenic *Leptospira* spp. However, they have some limitations with respect to specificity, sensitivity, reproducibility, and simplicity. LAMP is one potential method to resolve these limitations. The present study, *lipL32* gene is used as a target of LAMP detection. The gene encodes a protein that conserve among pathogenic and absence in non-pathogenic leptospires. The *lipL32* LAMP showed higher specificity than *rrs* LAMP[5,13,14,21] which primers were designed from 16S rRNA gene of pathogenic *Leptospira* spp. The *lipL32* LAMP detected the *lipL32* gene of pathogenic leptospires such as *L. interrogans* serovar Autumnalis, Bataviae, Pyrogenes, Javanica, Icterohaemorrhagiae, and Saigon. However, it did not detect DNA from *L. biflexa* serovar Patoc, human, *E. coli* and other bacteria included *Streptomyces* spp. SWU10 and *M. tuberculosis* (data not shown). Detection of clinical samples which had been identified bacterial strains revealed that *lipL32* LAMP can be detected both of pathogenic and intermediate group of *L. interrogans* including serovar Autumnalis, Pyrogenes, Hebdomadis, Grippotyphosa, Medanensis, Bataviae, Wolffi, Medanensis, and Pomona. Moreover, the sequences of all four LAMP primers showed 100% identity with at least 29 serovars of pathogenic *Leptospira* spp. implied of its specificity and applicability in detecting pathogenic leptospires in

Table 2. Serovars of *L. interrogans* that showed 100% identity with *lipL32* LAMP primers

No.	Serovar/strain	No.	Serovar/strain	No.	Serovar/strain
1	Balico	11	Icterohaemorrhagiae	21	Bratislava
2	Hardjo	12	Copenhageni	22	Veldrat Batavia
3	Autumnalis	13	Pomona	23	Perepelicin
4	Lai	14	Javanica	24	Sejroe
5	Weerasinghe	15	Hardjo-bovis	25	Paidjan
6	Canicola	16	Pyrogenes	26	Bataviae
7	Jalna	17	Wolffi	27	Bangkoki
8	Grippotyphosa	18	Tarassovi	28	Saigon
9	Hebdomadis	19	Australis	29	Shermani
10	Mini	20	Ballum		

clinical samples. Sensitivity of *lipL32* LAMP was 0.02 pg/µl or 4 GE/reaction which was more sensitive than *rrs* LAMP (10 GE/reaction)[5] and *lipL41* LAMP (100 GE/reaction)[8]. In addition, observation of fluorescence dye from LAMP product by the naked eye showed the same sensitivity as agarose gel electrophoresis. Therefore, the *lipL32* LAMP can be used to detect the early phase of infection without sign and symptom and can differentiate leptospirosis from the other infections with similar symptom.

In previous study, real-time PCR had been reported to detect the *lipL32* gene of pathogenic leptospires specifically[19,21,22]. The lower limit of detection were 3 GE/reaction in blood and approximately 10 GE/reaction in human urine[21] which comparable to our developed *lipL32* LAMP assay. However, the *lipL32* LAMP amplification has more advantage because it performed under isothermal conditions at 65°C within 1 h by using a simple and inexpensive device such as water bath or heat block, and can be diagnosed by the naked eye, therefore, it can be applied to the lab in endemic area of leptospirosis.

Conclusion

The developed *lipL32* LAMP method is high specificity and sensitivity that can be applied to detect pathogenic leptospire in clinical samples.

What is already known on this topic ?

The LAMP method has been developed for detections of pathogenic leptospires including 16S rRNA (*rrs*LAMP) and *lipL41* gene (*lipL41* LAMP).

The *rrs* LAMP exhibited low specificity; it was not recommended for diagnosis of clinical sample.

Real-time PCR assay using a TaqMan probe targeting *lipL32* detected 20 genomic equivalents/ reaction.

What this study adds ?

The developed *lipL32* LAMP method is high in specificity and sensitivity and can be applied to detect pathogenic leptospire in clinical samples.

Sensitivity of *lipL32* LAMP was 0.02 pg/µl or 4 GE/reaction; it was more sensitive than *rrs* LAMP (10 GE/reaction) and *lipL41* LAMP (100 GE/reaction).

The *lipL32* LAMP has more advantage than the other PCR techniques because it performed under isothermal conditions at 65°C within 1 h by using a simple and inexpensive device and can be diagnosed by the naked eye; therefore, it can be applied in the lab in endemic areas of leptospirosis.

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Potential conflicts of interest

None.

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การตรวจวินิจฉัยเล็บโตสไปร่าก่อโรคด้วยเทคนิค Loop-mediated isothermal amplification โดยใช้ lipL32 เป็นจีนเป้าหมาย

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ภูมิหลัง: โรคเล็ปโตสไปโรซิสหรือโรคฉี่หนูเป็นโรคระบาดซ้ำที่เกิดจากเชื้อเล็ปโตสไปร่าสายพันธุ์ก่อโรค ได้แก่ เล็บโตสไปร่า อินเทอโรแกนส์ (Leptospira interrogans)

วัตถุประสงค์: พัฒนาเทคนิค loop-mediated isothermal amplification (LAMP) เพื่อตรวจวินิจฉัยการติดเล็บโตสไปร่า อินเทอโรแกนส์ โดยใช้ lipL32 เป็นจีนเป้าหมาย

วัสดุและวิธีการ: ไพรเมอร์สองคู่ออกแบบให้จำเพาะต่อส่วนที่อนุรักษ์ของจีน lipL32 ของเล็ปโตสไปร่าสายพันธุ์ก่อโรคหลายชนิด สำหรับปฏิกิริยาของ LAMP ทำที่ 65 องศาเซลเซียส เป็นเวลา 1 ชั่วโมง ตรวจสอบผลผลิตของ LAMP ด้วยวิธีอะกาโรสเจลอิเล็คโตรโฟริซิสและการใช้สีฟลูออเรสเซนซ์

ผลการศึกษา: ผลการทดลองพบว่าปฏิกิริยา LAMP มีความจำเพาะต่อ เล็บโตสไปร่า อินเทอโรแกนส์ ซีโรวาร์ Autumnalis, Bataviae, Javanica, Pyrogenes, Icterohaemorrhagiae และ Saigon ไม่มีปฏิกิริยากับเล็ปโตสไปร่าสายพันธุ์ไม่ก่อโรค (L. biflexa), เชื้อจุลินทรีย์ชนิดอื่น เช่น Escherichia coli และดีเอ็นเอของคน ความไวของเทคนิคนี้เมื่อวิเคราะห์ผลผลิตของ LAMP ด้วยอะกาโรสเจลอิเล็กโตรฟอรีซิส และการสังเกตสีฟลูออเรสเซนซ์ด้วยตาเปล่า เท่ากับ 0.02 pg/μl ซึ่งเท่ากับปฏิกิริยานี้สามารถตรวจหาเชื้อเล็บโตสไปร่าสายพันธุ์ก่อโรคได้ เมื่อมีจีโนมของเชื้ออย่างน้อย 4 จีโนม นอกจากนี้เทคนิคนี้สามารถตรวจหาเล็ปโตสไปร่าสายพันธุ์ที่แยกจากคนไข้ ซึ่งประกอบด้วยเล็ปโตสไปร่าสายพันธุ์ก่อโรคและกลุ่ม intermediate ได้อีกด้วย

สรุป: เทคนิค lipL32 LAMP ที่พัฒนาขึ้นนี้สามารถนำไปประยุกต์ใช้ในการตรวจวินิจฉัยเล็ปโตสไปร่าสายพันธุ์ก่อโรคในคนไข้