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# การพัฒนาวิธี LAMP-Lateral Flow Immunoassay สำหรับการวินิจฉัยโรคเมลิออยด์

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# Development of LAMP-Lateral Flow Immunoassay for Diagnosis of Melioidosis

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## บทคัดย่อ

**หลักการและวัตถุประสงค์:** โรคเมลิออยด์เป็นโรคร้ายแรงเกิดจากการติดเชื้อแบคทีเรียแกรมลบ *Burkholderia pseudomallei* ผู้ป่วยที่ติดเชื้อในกระแสเลือดอาจเสียชีวิตได้อย่างรวดเร็วภายใน 48 ชั่วโมง ดังนั้นการตรวจวินิจฉัยอย่างรวดเร็วจึงมีความสำคัญ วัตถุประสงค์ของการศึกษานี้เพื่อพัฒนาเทคนิคการตรวจหาเชื้อด้วยวิธีลูปเมดิเอเตท ไอโซเทอร์มอลแอมพลิฟิเคชั่น (LAMP) ร่วมกับเทคนิคแลทเทอรอลโฟลว์ ดิปสติก (LFD) เพื่อการตรวจหาเชื้อ *B. pseudomallei*

**วิธีการศึกษา:** เลือกจีน *wcbG* ซึ่งเป็นยีนที่คาดการณ์ว่าเกี่ยวข้องกับการสร้างโปรตีน capsular polysaccharide protein ของเชื้อ *B. pseudomallei* มาออกแบบไพรเมอร์ สำหรับวิธี LAMP จำนวน 4 ไพรเมอร์ และ 1 โพรบ โดยอาศัยโปรแกรม Primer Explorer software ทำการทดลองหาสภาวะที่เหมาะสม สำหรับวิธี LAMP-LFD และศึกษาความไว (sensitivity) ของวิธี LAMP-LFD โดยใช้ *B. pseudomallei* DNA ปริมาณตั้งแต่ 50 นาโนกรัม ถึง 500 เฟมโตกรัม ตลอดจนประเมินความจำเพาะด้วยการทดสอบกับ DNA ของเชื้อแบคทีเรียปริมาณ 50 นาโนกรัม จำนวน 12 สายพันธุ์ หลังจากนั้นได้ประเมินความสามารถในการตรวจหาเชื้อ *B. pseudomallei* ในเลือดที่มีสารกันเลือดแข็ง EDTA ที่มีเชื้อปริมาณตั้งแต่ $10^6$ CFU ถึง 1 CFU

**ผลการศึกษา**: จากการศึกษาพบว่าผู้วิจัยสามารถพัฒนาชุดตรวจ LAMP-LFD ได้สำเร็จ โดยใช้ 1M betaine และสภาวะที่อุณหภูมิ 60°C เป็นเวลา 60 นาที สำหรับรอบของ LAMP และใช้อุณหภูมิ 60°C เป็นเวลา 5 นาที สำหรับการทำ hybridization กับ FITC-probe LAMP-LFD ให้ความไวที่ ปริมาณ 5 พิโคกรัมต่อมิลลิลิตร และความจำเพาะ 100% เมื่อทดสอบกับเชื้อ *B. thailandensis*, *Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli*, *Salmonella* group B, *Pseudomonas aeruginosa* และ *Klebsiella pneumonia* ปริมาณต่ำสุดที่สามารถตรวจได้ในเลือดที่มีสารกันเลือดแข็ง EDTA คือ $10^2$ CFU ต่อเลือด 200 ไมโครลิตร

**สรุป:** ชุดตรวจ LAMP-LFD ที่พัฒนาขึ้นโดยใช้ยีน *wcbG* ให้ความไว และความจำเพาะสูงสำหรับการตรวจหาเชื้อ *B. pseudomallei* ในเลือด งานวิจัยนี้จึงเป็นอีกวิธีทางเลือกหนึ่งของการตรวจวินิ จฉัยโรคเมลิออยด์อย่างรวดเร็ว

**คำสำคัญ:** โรคเมลิออยด์, การวินิจฉัยทางห้องปฏิบัติการ, แลมป์, แลทเทอรอลโฟลว์ ดิปสติก

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## Abstract

**Background and Objective**: Melioidosis is a fatal disease caused by a gram-negative bacterium, *Burkholderia pseudomallei.* Patients with septicemic melioidosis die within 48 hours, the rapid diagnosis using blood samples is essential. The aim of this study was to develop a loop-mediated isothermal amplification (LAMP) combined with a lateral flow dipstick (LFD) assays for the detection of *B. pseudomallei*.

**Methods**: The *wcbG* gene, a putative capsular polysaccharide biosynthesis protein gene of *B. pseudomallei* was selected for LAMP primers design. Four primers and 1 probe were considered using Primer Explorer software. The conditions for LAMP-LFD were optimized. The sensitivity of LAMP-LFD was investigated with 50 ng to 500 fg of *B. pseudomallei* genomic DNA while the specificity was evaluated by using 50 ng of 12 bacterial strains. The LAMP-LFD method was evaluated by the spiked $10^6$ CFU to 1 CFU of *B. pseudomallei* into the normal EDTA blood samples.

**Results:** The LAMP-LFD was successfully developed using 1M betaine, 60°C for 60 min for LAMP cycles and conditions for hybridization with FITC-probe were at 60°C for 5 min. It gave sensitivity of 5 pg/ml and 100% specificity when tested with *B. thailandensis*, *Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli*, *Salmonella* group B, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*. The detection limit was genomic DNA of $10^2$ CFU per 200 µl of EDTA blood.

**Conclusion**: Our LAMP-LFD assay provided a highly sensitive and specific method to detect *B. pseudomallei* in the blood based on *wcbG* gene. It will be another approach for rapid diagnosis of melioidosis.

**Keywords**: Melioidosis, Laboratory diagnosis, LAMP, LFD.

## Introduction

*Burkholderia pseudomallei* is a gram-negative bacterium and the causes of a disease called melioidosis. Infection can be occurred via ingestion, cutaneous or airborne routes. The disease can occur both acute and chronic forms[1]. In Thailand, this disease is the second most common cause of community-acquired bacteremia and mortality rate about 43% especially in Northeast Thailand[2]. The current laboratory diagnosis is culture, as gold standard method. The culture method is required for 3-7 days before the results come out[1]. These processes are time-consuming and the need for skilled isolation. Therefore, early diagnosis is necessary for patients with sepsis. Several serological tests are used for diagnosis of melioidosis such as IHA, IFA, ELISA and latex agglutination. They still are not appropriate as low sensitivity are still encountered and the result does not correlate with clinical finding. Molecular diagnosis such as PCR is also developed and gives lower sensitivity than culture. In 2014, Houghton et al[3]. reported the prototype active melioidosis detect lateral flow immunoassay (LFI) used for detection of *B. pseudomallei* capsular polysaccharide (CPS) in melioidosis patient samples. The results of LFI demonstrated that analytical specificity was 97.2% (35/36) and the limit of detection was ≈ 0.2 ng/ml. In 2018, Wongsuvan et al. found that LFI detect *B. pseudomallei* CPS in serum samples with the sensitivity of 31.3% and the specificity of 98.8%. The result suggested that LFI may be limited sensitivity when testing in the blood samples[4]. Loop-mediated isothermal amplification assay or LAMP is an alternative technique of DNA amplification under isothermal conditions. This was developed by Notomi et al. in 2000[5]. LAMP is a simple, rapid, sensitive, specific and cost-effective technique. Which does not need a thermal cycler and can be performed in a water bath or heat block. In 2008, Chantratita et al[6] showed the LAMP assay used for the detection of *B. pseudomallei* based on targeting the TTS1 gene cluster. They demonstrated that the limit of detection was 38 genomic copies per reaction and LAMP was positive for all *B. pseudomallei* isolates but not for the closely related species. However, the sensitivity for detection of patients with suspected melioidosis was 44%, and the result could be of low sensitivity.

In this study, we aimed to develop a rapid, sensitive, and specific probe-based LAMP assays with lateral flow immunoassay (LFI) for the rapid detection of *B. pseudomallei.* The primer sequences were designed base on the *wcbG* gene. Although this gene was also found in *B. mallei*, it was designed specifically in *B. pseudomallei* and it did not present in closely related species of bacteria such as *B. thailandensis* or *B. cepacia*[7].

## Materials and Methods

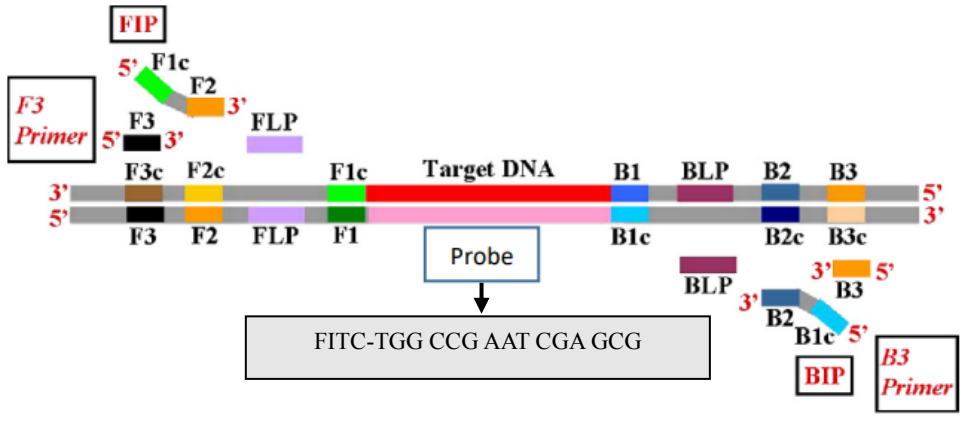
### Bacterial strains and DNA extraction

Bacterial strains used for the spiking experiment in this study are 4 strains of *B. pseudomallei* involved in clinical isolated from blood patients with septicemic melioidosis including *B. pseudomallei* K96243, a standard strain, and 844, 1909A, 1026b. All strains of *B. pseudomallei* were obtained from the Melioidosis Research Center (MRC), Khon Kaen University, Khon Kaen, Thailand. Six clinical strains of other microorganism involved in sepsis in the hospitals were also used as a negative control i.e., *Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli*, *Salmonella* group B, *Pseudomonas aeruginosa* and *Klebsiella pneumonia.* The closely related strains of *B. pseudomallei* including two environmental strain of *B. thailandensis* UE4 and E264 were also included. All microorganism that were used as a negative control were kindly provided by Asst. Prof. Dr.Umaporn Yordpratum, Faculty of Medicine, Khon Kaen University. Their genomic DNA extraction was done by the method described by Anderson and McKay with some modifications.[14]

### *B. pseudomallei*-specific target DNA sequence for LAMP primers and probe

LAMP primers were designed based on the *B. pseudomallei wcbG* gene (accession number CP002833), a putative capsular polysaccharide biosynthesis protein gene sequence[7] using Primer Explorer software (https://primerexplorer.jp/e/). The primer set comprised of four primers. The two outer primers, forward outer primer (F3) and backward outer primer (B3); two inner primers, forward inner primer (FIP) and backward inner primer (BIP) were used to perform LAMP and LAMP-LFD assay. The primer binding regions, their orientations and nucleotide sequence of the target region are shown in Figure 1.

A




B

| Primer name | Nucleotide sequence (5′– 3′) |
|---|---|
| F3 | CGA ACG ATC GAT TCC GTC |
| B3 | CGG AGC GAT CCA ATG TTC A |
| Biotin-FIP | Biotin-CAG TAG ACA ATC TTC TCC GAA TTT TAG TCG GTC ATT TCC CTG ACG G |
| BIP | AAG GAC GTA CCG AGC GTC TTT ATT TTC GGC AAA AAA ATG CTT GA |
| FITC-Probe | TGG CCG AAT CGA GCG |

C

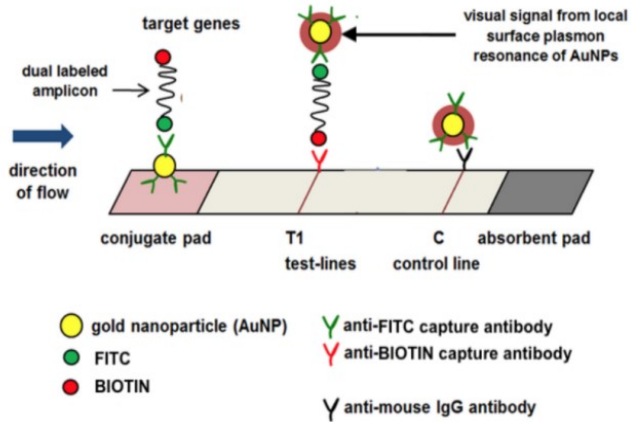



**Figure 1** LAMP-LFD design. The schematic diagram of two-inner (FIP and BIP) and two–outer (F3 and B3) primers and FITC-probe for LAMP assay (A) and the primer sequences used in this study (B). The detection of LAMP product by LFD are shown in C.

### LAMP optimization

For optimizations, fifty nanograms of genomic DNA of *B. pseudomallei* K96243 were used in each reaction and nuclease-free water as negative control. LAMP assay was done in various concentrations of betaine at 0.1, 0.25, 0.5 and 1 M. The LAMP amplification was performed in 30, 45 and 60 min, while the effect of temperature was determined at 60, 63 and 65 °C. The amplified products (8 µl) were detected by 2.5% agarose gel electrophoresis followed by ethidium bromide staining. The condition that showed ladder-like DNA was considered.

### LAMP conditions

The final reaction optimized conditions were as follows: the LAMP reaction was carried out in a 25 µl mixture containing 10X ThermoPol Buffer (including 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, 0.1 % Triton X-100, pH 8.8 @ 25°C, NEB company, New England Biolabs, UK) 2.5 µl, 100 mM

MgSO4 (NEB company, New England Biolabs, UK) 1.5 µl, 10 mM dNTPs (Invitrogen by Thermo Fisher Scientific, USA) 3.5 µl, *Bst* 2.0 WarmStart DNA polymerase (8,000 U/ml) (NEB company, New England Biolabs, UK) 1 µl, 40 µM inner primers (Pacific Science Co, Ltd., Bangkok, Thailand) 1 µl, 5 µM outer primers (Pacific Science Co, Ltd., Bangkok, Thailand) 1 µl, positive template 2 µl, nuclease-free water was used as the template in the negative control sample 7.5 µl.

### Lateral flow dipstick (LFD) assay

To detect the LAMP products of biotin labeling, the hybridization with labelled probe was performed. The LAMP products were added to the sample pad of the LFD strip and migrated by capillary action, where the biotin LAMP product hybridized with a FITC-probe a complexed with gold-labeled anti-FITC antibody (Figure 1C).

Following to the assay protocol, the reaction mixtures containing LAMP amplicons 10 µl and 20 µM FITC-DNA probe 1 µl were incubated at 60°C for 5, 10 or 15 min. Subsequently, 10 µl of hybridized product was added to 100 µl of running buffer (Serve Science Co., Ltd., Bangkok, Thailand) in a new tube. An LFD (Serve Science Co., Ltd., Bangkok, Thailand) was dipped into the reaction mixture for 5 min. A red-purple line was observed at the control line for all strips, which confirmed that the test was correctly operated.

### Sensitivity and specificity of LAMP–LFD

The sensitivity of LAMP-LFD was investigated with different concentrations of genomic DNA of *B. pseudomallei* K96243 was used as DNA templates for biotin labeling LAMP tests performed under optimized conditions and nuclease-free water as negative control. Ten-fold serial dilutions of genomic DNA *B. pseudomallei* K96243 including 50 ng, 5 ng, 500 pg, 50 pg, 5 pg and 500 fg were incubated at 60°C for 60 min. The specificity of LAMP-LFD assay was evaluated by using 50 ng of different bacterial strains. In this study, genomic DNA including *B. pseudomallei* (strain K96243, 844, 1026b and 1909A), *B. thailandensis* (strain UE5 and E264), *Escherichia coli* ATCC25922, *Salmonella* group B, *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *S. epidermidis.* Ten µl of each LAMP amplicons and 1 µl of 20 µM FITC-DNA probe were incubated at 60°C for 5 min. After that, 10 µl of hybridized product was added to 100 µl of running buffer (Serve Science Co., Ltd., Bangkok, Thailand) in a new tube. For detection, an LFD was dipped into the reaction mixture for 5 min. All LAMP amplicons were analyzed using the LFD assay.

### Detection of *B. pseudomallei* in spiked inoculated blood samples

To demonstrate the ability of LAMP-LFD assay as a detection tool for diagnosing *B. pseudomallei* in inoculated blood samples, 200 µl of human EDTA blood was spiked with either $10^6$ CFU to 1 CFU of *B. pseudomallei* K96243. To determine the concentration, ten-fold serial dilutions of *B. pseudomallei* K96243 culture including $10^6$ CFU to 1 CFU were measured at 600 nm and determined the number of bacteria (CFU/200 µl) on LB agar by drop plate method. Genomic DNA from the spiked blood samples were isolated using DNA extraction according to the manufacturer's protocol with some modifications[9]. The samples were used in LAMP-LFD reaction. Genomic DNA isolated from normal blood samples was used as negative control.

## Results

### Optimization of LAMP and LAMP-LFD for detection of *B. pseudomallei*

LAMP-LFD for detection of *B. pseudomallei* was successfully developed. The optimal conditions are shown in Figure 2. As betaine is important for LAMP, the results showed that 1 M of betaine gave the best amplification result (Figure 2A). The optimal reaction temperatures and times were further investigated. The reaction mixture was tested at 60, 63 or 65°C for 30, 45 or 60 minutes. The results demonstrated that the best conditions for LAMP method of *B. pseudomallei* K96243 required 60°C for 60 min (Figure 2B). After amplification, we analyzed LAMP products using a lateral flow dipstick.

The DNA probe for hybridization with LAMP amplicons to LFD detection was performed. In order to determine the optimal time of hybridization. The LAMP products were immediately mixed with 1 µl of 20 µM of FITC-DNA probe followed by further incubation at 60°C varied from 5, 10 or 15 min to allow hybridization to take place. The results demonstrated that all three times evaluated gave similar LFD patterns at 60°C (Figure 2C). We therefore used LAMP products hybridized with FITC-probe incubation at 60°C for 5 min for further test.

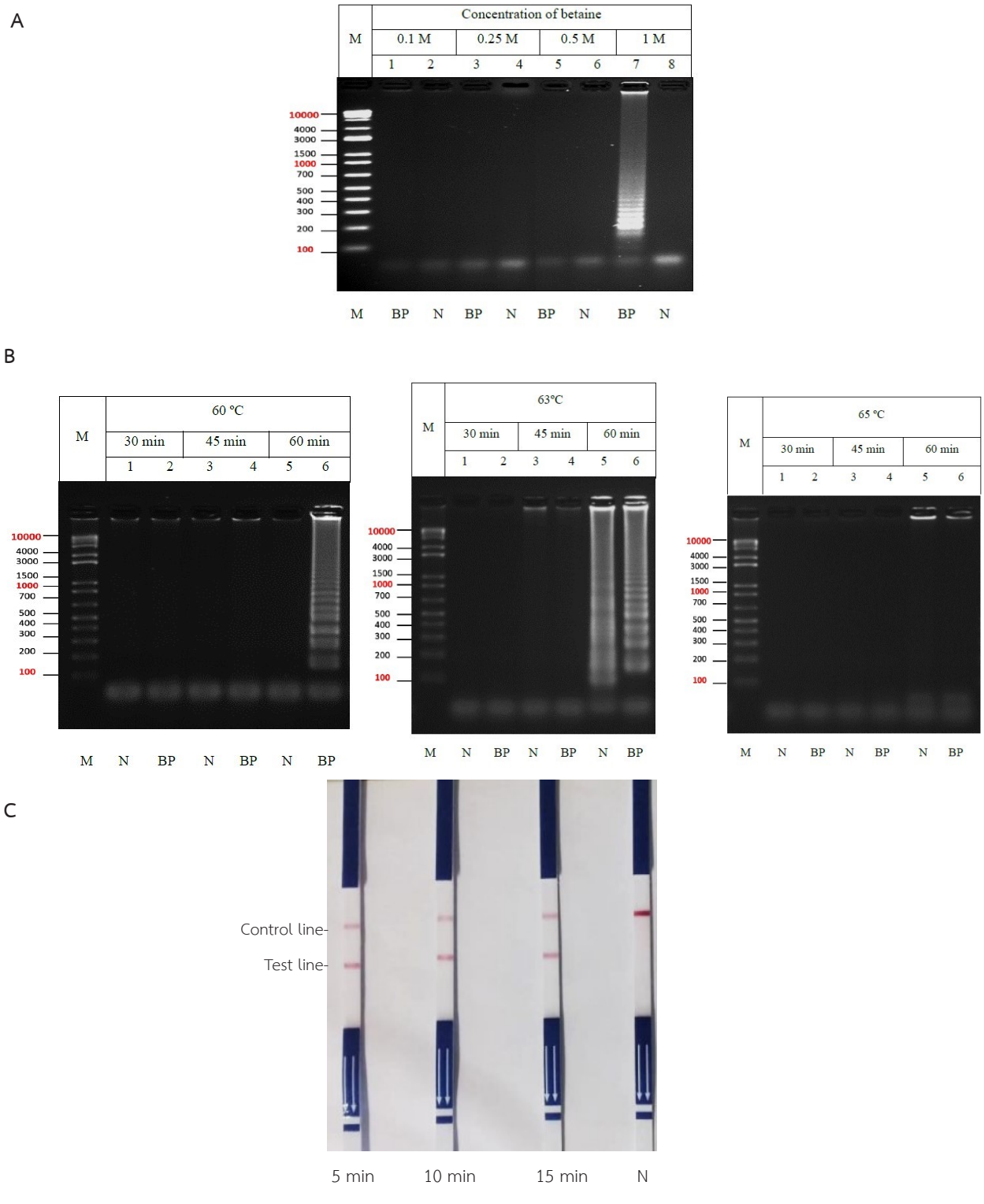



**Figure 2** Optimization of LAMP-LFD assay. Ethidium stained 2.5% agarose gel electrophoresis of LAMP products (A and B). The LAMP reaction contained 50 ng of *B. pseudomallei* DNA, 10X isothermal buffer, outer primers (F3 and B3), inner primers (FIP and BIP), dNTPs, $MgSO_4$ and *Bst* 2.0 WarmStrat DNA polymerase were used following a standard protocol as described earlier (New England Biolabs® inc). Panel A is the optimization of concentration of betaine while B is the optimization of times and temperature for the LAMP reactions. Lane M is 1 Kb plus DNA ladder. Lanes BP are LAMP products from genomic DNA of *B. pseudomallei* (BP) while lanes N are DW as negative control. Panel C is the dipstick-DNA chromatography of LAMP products (LFD). LAMP products from 50 ng of *B. pseudomallei* gDNA hybridized with FITC-probe and detected using lateral flow dipsticks via incubation at 60°C for 5, 10 and 15 min (strips 1-3) respectively. Strip 4 (N) is distilled water as the negative control.

**Sensitivity and specificity of LAMP-LFD**

We performed sensitivity analysis of biotin labeling LAMP tests using different concentrations of *B. pseudomallei* DNA. The DNA were ten-fold serial dilutions from 50 ng to 500 fg in sterile distilled water and used for LAMP reaction. The reaction without DNA template was applied as the negative control. To determine the sensitivity, the amplified mixtures were analyzed by LAMP-LFD assay. We found that the lower limits of LAMP-LFD detections for *B. pseudomallei* was 5 pg as shown in Figure 3A.

The specificity of the DNA probe was examined using 50 ng templates for *B. pseudomallei* and other bacteria. Our results demonstrated no crossreactivity with other bacteria using the LAMPLFD assay was shown in Figure 3B.

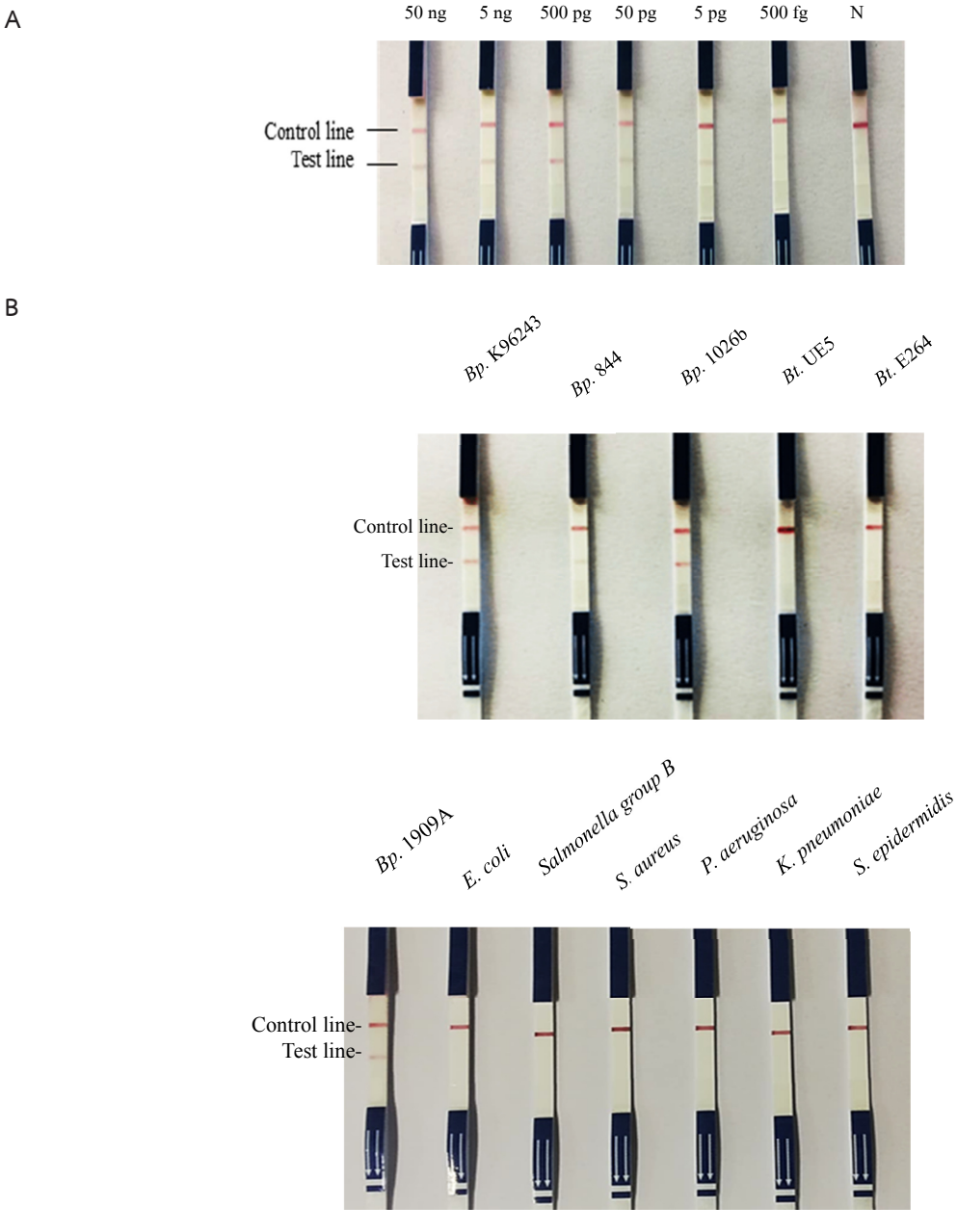



**Figure 3** Sensitivity and specificity of the LAMP-LFD. *B. pseudomallei* genomic DNA (500 fg-50 ng) were used as templates for LAMP (A). Genomic DNA (50 ng) from different bacterial species (B) were used as template for LAMP. The LAMP amplicons hybrid products were added on the LFD strip. Distilled water was used as a negative control (N).

**Detection of *B. pseudomallei* in artificial inoculated blood samples**

To test the efficacy of LAMP-LFD assay, different concentrations of the *B. pseudomallei* genomic DNA in spiked blood were tested using LAMP-LFD assay. The healthy normal blood was used as negative control. These results in figure 4 indicated that the detection limit of LFD was $10^2$ CFU per 200 µl of EDTA blood (Figure 4).

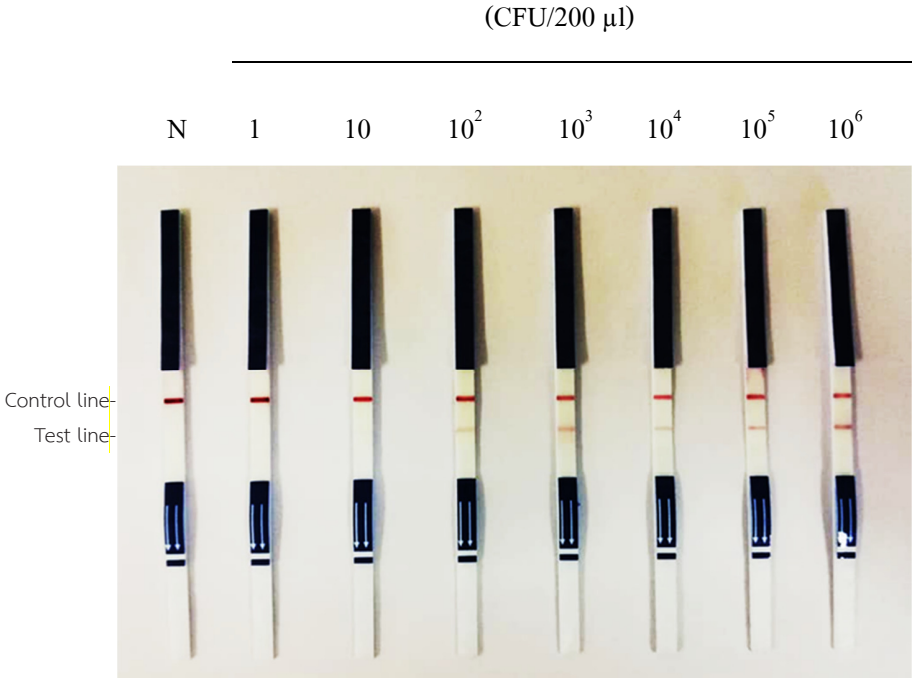



**Figure 4** Detection limit of LAMP-LFD assay using naked eye observations of a red band in the test line. The healthy EDTA blood (200 µl) were spiked with 1-$10^6$ cells and used for LAMP. After blood samples were processed, 5 ul of DNA was used in LAMP-LFD. N is negative control containing distilled water.

## Discussion

Melioidosis is an important disease caused by the Gram-negative bacterium *B. pseudomallei.* Since the clinical symptoms give broad ranges making the diagnosis of patients is difficult. Laboratory diagnosis of melioidosis is based on either culture of pathogen from specimen or a rise in serum antibody. In many cases, patients with acute and severe septicemic melioidosis died from delayed of diagnosis and appropriate antibiotic treatment[10-12]. The current identification for melioidosis is bacterial culture. This method remains as the gold standard, which requires 3 to 7 d for the processes of pre-enrichment or growth on selective culture medium. Moreover, isolation of *B. pseudomallei* from several contaminated sites for example in fecal, pus or sputum specimens, can be difficult[13,14]. Several serological tests have been developed including the indirect hemagglutination assay (IHA), the enzyme-linked immunosorbent assay (ELISA) and a lateral-flow dipstick (LFD)[6].

Our study aimed to develop a more rapid DNA based method for the detection of *B. pseudomallei*. The direct detection methods in human blood is difficult as the low number of bacteria (1-10 cells/ml) found in infected patients[15]. In order to make the methods more sensitive and rapid, Loop-mediated isothermal amplification (LAMP) assay was used to rapid amplification of specific DNA targets. Moreover, to increase the detection steps and speed up the result time, lateral flow dipstick (LFD), a simple and rapid method were combined with LAMP. The LFD are simple and can also be visualized by eye.

The target gene used for LAMP is *wcbG* gene. We used this gene as it is capsular polysaccharide biosynthesis protein localized in cytoplasm. This gene was found only in pathogenic bacteria in only 1 genus, *Burkholderia*. The primer sequences given were

checked for specific using Blastn confirm that homology among the *B. pseudomallei* strain. The specific of this test depended on specific primer pair, we designed a set of primers based on *wcbG* gene which reference the gene[16]. LAMP technique has been widely applied in many fields, especially in molecular diagnostics with a sensitive and rapid detection technique. Various detecting techniques, such as colorimetric, turbidity, fluorescent agents, agarose gel electrophoresis and LFD, have been employed for analysis of LAMP amplicons[17,18]. Although LAMP had been used for *B. pseudomallei* detection[6] the sensitivity was low and it needs more time to run on the gel for detection.

In our study, a rapid and efficient LAMP combined with LFD assay for detecting *B. pseudomallei* was developed. It was suitable for used in hospital, simple with a low requirement in experimental conditions and did not require additional or expensive laboratory equipment. Our LAMP protocol mentioned in this study, the optimization of LAMP condition demonstrates a high sensitivity with the lower limits of detections for *B. pseudomallei* was 5 pg of genomic DNA target per reaction. When specificity was a concern, the results showed that no cross-reactivity between *B. pseudomallei* and other bacterial strains using the LAMP-LFD assay.

In 2008, Chantratita et al[6] demonstrated that the LAMP assay for the detection of *B. pseudomallei* was highly sensitive and specific based on targeting the TTS1 gene cluster for diagnosis of melioidosis. They demonstrated that incubation at temperature of 65°C and time of 90 min gave the greatest observable turbidity by eye. It was confirmed by the visualization of LAMP products using gel electrophoresis. Moreover, the limit of detection was 38 genomic copies per reaction, and LAMP was positive for all *B. pseudomallei* isolates but not for the closely related species. In 2018, Liu et al[19]. demonstrated that when the reaction parameters of LAMP were optimized, LAMP products could be applied for detection by three methods including SYBR Green I staining, lateral flow dipstick (LFD) and agarose gel electrophoresis. They described that LAMP-LFD was suitable for field-based detection of Spring viremia of carp virus (SVCV) with its advantages of speed, simplicity, and disposability. Several researchers reported LAMP products hybridized to specific probes and subsequently detected using LFD[19-22]. It is sufficient for the detection of hybridized LAMP products and the results are visualized by eye with easy to detect without the use of carcinogens, such as ethidium bromide[21,23-25].

## Conclusion

Our LAMP-LFD assays provided a rapid and simple method to detect of low amount of *B. pseudomallei* in the EDTA blood based on *wcbG* gene. Further application for clinical samples remains to be evaluated.

## Acknowledgements

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