Development of Rapid Detection for *Burkholderia pseudomallei* by Loop-mediated Isothermal Amplification

การพัฒนาตรวจหาเชื้อ Burkholderia pseudomallei อย่างรวดเร็วโดยเทคนิค loop-mediated isothermal amplification

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ABSTRACT

The present study was aimed to establish a loop-mediated isothermal amplification (LAMP) technique for the rapid detection of *Burkholderia pseudomallei*, the causative agent of melioidosis. A set of six specific primers targeting type III secretion system 1 (TTS1) were designed for the LAMP test. The reaction was optimized using different reaction temperature and Mg^{2+} concentrations. The detection limit of LAMP assay was 10 pg/µL and no cross-reaction was observed, indicating that LAMP assay has high specificity. The assay showed successful detection of *B. pseudomallei* in clinical samples. Using culture method as gold standard, the sensitivity, specificity, positive predictive values, negative predictive values of LAMP were 100%. In conclusion, LAMP assay is a high efficiency, low cost diagnostic tool, useful for rapid, accurate, direct detection of *B. pseudomallei* for clinical diagnosis.

บทคัดย่อ

ในการศึกษานี้มีวัตถุประสงค์เพื่อจัดตั้งเทคนิค loop-mediated isothermal amplification (LAMP) เพื่อตรวจหาเชื้อ *Burkholderia pseudomallei* อย่างรวดเร็ว ซึ่งเชื้อดังกล่าวเป็นสาเหตุของการเกิดโรค melioidosis ในปฏิกิริยาแลมป์ประกอบไปด้วยไพรเมอร์จำนวน 6 เส้นที่ออกแบบโดยอ้างอิงยีน type III secretion system 1 (TTS1) การศึกษาสภาวะที่เหมาะสมสำหรับปฏิกิริยาประกอบไปด้วยอุณหภูมิและความเข้มข้นของ $MgSO_4$ ที่เหมาะสม ผลการศึกษาแสดงให้เห็นว่าปฏิกิริยาแลมป์มีค่า detection limit เท่ากับ 10 pg/ µL โดยไม่พบปฏิริยาข้ามระหว่างแบคทีเรียสายพันธ์อื่น แสดงให้เห็นว่าปฏิกิริยาที่จัดตั้งขึ้นมีความไวและความจำเพาะ ในการทดสอบกับสิ่งส่งตรวจทางคลินิกโดยเปรียบเทียบผลกับการเพาะเลี้ยงเป็นวิธีมาตรฐานพบว่าเทคนิคแลมป์ที่จัดตั้งมีความไว ความจำเพาะ ค่าทำนายผลบวก ค่าทำนายผลลบเท่ากับร้อยละ 100 ผลการศึกษานี้สรุปได้ว่าเทคนิคแลมป์เป็นวิธีที่มีประสิทธิภาพ เหมาะสมในการพัฒนาเพื่อต่อไป

Keywords: *Burkholderia pseudomallei*, LAMP

คำสำคัญ: *Burkholderia pseudomallei* แลมป์

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Introduction

B. pseudomallei, a gram-negative bacillus, is the agent of melioidosis which is endemic in Southeast Asia and Northeast Australia. It is commonly found in soil and water, and it has been isolated from a variety of clinical samples in endemic areas (Paiva, et al., 2017). Besides Southeast Asia and Northeast Australia, melioidosis has been reported in the South Pacific, Africa, India, the Middle East, Central America and South America (Samy, et al., 2017).

The diagnosis of *B. pseudomallei* is based on cultural, serological and molecular assays. Growth on standard blood culture commonly occurs in about 2-3 days. The effective molecular assays with high accuracy based on PCR have been also intensively developed in recent years (Samy, et al., 2017). However, these methods have some limitations such as high cost and need of specialized equipment especially in developing countries where the disease remains endemic (Aziz, et al., 2017). Laboratory diagnostic is important not only for the prescribing of effective drugs for appropriate patients in adequate doses for treatment the person in case but also for preventing the evolution of the antibiotic resistance of *B. pseudomallei.*

Loop-mediated isothermal amplification (LAMP) is an alternative method for rapid DNA amplification under isothermal condition. This method employs a DNA polymerase and set of four or six specially designed primers. The cycling reaction results in the accumulation of 10^9 copies of target in less than one hour (Rongkard, et al., 2016). The assay is quick and easy to perform, and all it requires is a laboratory water bath or heat block that maintains a constant temperature of 60 to 65°C (Kohler, et al., 2016).

Objectives of the study

The aim of this study was to develop a sensitive and specific LAMP method for detection of *B. pseudomallei* and compare this assay to a cultural technique for diagnosis of melioidosis.

Materials and Methods

Bacterial strains and clinical specimens

In this study, LAMP was initially developed by using *B. pseudomallei* standard strain (ATCC 23343) which kindly provided by Melioidosis Research Center, Faculty of Medicine, Khon Kaen University, Thailand. DNA extraction was performed by a loopful culture dissolved in a microtube containing 400 µL TE buffer (10 mM Tris/HCl (pH 8.0), 1 mM EDTA). The mixture was heated at 95°C for 10 min, centrifuged at 12000 rpm for 15 min and the supernatant was used as positive control for LAMP optimization.

A total of 91 clinical specimens from patients admitted to Sanprasitthiprasong Hospital, Ubon Ratchathani, Thailand were collected and extracted using GF-1 DNA Extraction Kit (Vivantis Technologies, USA). All DNA samples were stored at -20°C until used. All samples were first diagnosed by culture and then evaluated by LAMP assay.

Primers design for LAMP

The strain-specific primers for LAMP assay of *B. pseudomallei* were designed based on TTS1 gene (GenBank No. AF074878) by using the Primer Explorer V4, LAMP primer designing software (https://primerexplorer.jp/e/). This region is only found in *B. pseudomallei* but not *B. mallei* and *B. thailandensis* (Price, et al., 2016). The sequence of each primer was shown in Table 1.

Optimization of LAMP assay

Optimizing of LAMP condition for *B. pseudomallei* was determined by amplifying 100 ng/µL of positive control. The reaction was carried out in a final volume of 25 µL. Briefly, the reaction mixture contained 0.2 µM of each outer primer (F3, B3), 1.6 µM of each inner primer (FIP, BIP), 0.8 µM of each loop primer (LF, LB), 8 units of *Bst* DNA polymerase (New England Biolabs, USA) and 1X supplied buffer along with different reaction temperatures (57, 59, 61, 63 and 65°C) and $MgSO_4$ concentrations (1, 2, 3, 4, 5, 6, 7 and 8 mM). The LAMP products were analyzed on a 2% agarose gel electrophoresis.

Detection limit of LAMP assay

To determine the detection limit of LAMP assay, positive genomic DNA was serially 10-fold diluted ranging from 200 ng/µL to 10fg/µL, the detection limit was analyzed based on the least DNA concentration which gave band on agarose gel electrophoresis.

Specificity of LAMP assay

The specificity of LAMP was examined using 100 ng of total DNA extracted from *B. mallei, B. thailandensis, Klebsiella pneumonia, Pseudomonas aeruginosa, Salmonella sp, Shigella sp and E.Coli* and LAMP reactions were undertaken with optimized conditions.

Evaluation of LAMP with clinical specimens

To evaluate LAMP assay in clinical samples, a total of 91 samples including blood, sputum and pus were firstly tested by culture. All samples were then subjected to LAMP assay. The analytical sensitivity, specificity, positive predictive value and negative predictive value of LAMP were calculated using standard formulas based on culture results as gold standard.

Results

Optimal conditions for LAMP assay

To determine the optimal condition of *B. pseudomallei* detection, LAMP was conducted with different parameters including reaction temperature and Mg^{2+}. The presence and absence of the bands after electrophoresis was used to assess the optimum condition. On the basis of the above results, the LAMP condition was contained 0.2 µM of

each outer primer (F3, B3), 1.6 µM of each inner primer (FIP, BIP), 0.8 µM of each loop primer (LF, LB), 8 units of *Bst* DNA polymerase, 1X supplied buffer along with 5 mM $MgSO_4$. The amplification was optimal carried out at 63°C for 60 min and the reaction terminated by 80°C for 10 min. The reaction temperature at 63°C and 4 mM $MgSO_4$ were chosen as optimal condition for further study as shown in Figure 1 and 2.

Detection limit of LAMP assay

To determine the detection limit, positive DNA template was serially 10-fold diluted. The detection limit of LAMP was found to be 10 pg/µL as shown in Figure 3.

Specificity of LAMP assay

To determine the specificity, the 100 ng/µL of total DNA extracted from *B. pseudomallei* standard strain were used as DNA template. The results showed that DNA amplification was only observed when the primer set was reacted with *B. pseudomallei*. The positive reaction was clearly seen whereas no LAMP products were detected in reaction carried out with other organisms (Figure 4).

Evaluation of LAMP in clinical samples

Optimal LAMP assay was evaluated for detection of *B. pseudomallei* in clinical specimens. All samples used in this study were tested by culture method. The total 91 specimens were composed of 73 samples of *B. pseudomallei,* 3 samples of *B. mallei*, 10 samples of *B. thailandensis*, 1 sample of *Klebsiella,* 1 sample of *pneumonia,* 1 sample of *Pseudomonas aeruginosa,* 1 sample of *Salmonella sp,* 1 sample of *Shigella sp and* 1 sample of *E.Coli*. Using LAMP assay, the results was similarly detected as culture method; the sensitivity, specificity, positive predictive value and negative predictive value of LAMP assay were 100%. Thus, LAMP assay was effective in detecting *B. pseudomallei* in clinical samples (Table 2).

Discussion and conclusions

In order to have a successful management system of melioidosis and for having a full control of *B. pseudomallei* in term of the prescription of effective drugs for appropriate patients in adequate doses for treatment the person in case, for preventing the evolution of the antibiotic resistance of *B. pseudomallei*, for having an effective point of care laboratory testing applicable in resource-poor endemic areas, a reliable laboratory method for rapid detection of *B. pseudomallei* is required. Such detection method should play an important role in early diagnosis of this infection. LAMP represents a novel DNA amplification technique that can amplify bacterial DNA from clinical samples collected from patients infected by melioidosis. Our developed LAMP in this study can amplify DNA with high specificity, efficiency and rapidly under isothermal optimized conditions at 63°C for 60 minutes and is faster than PCR which require 2 to 3 hours excluding the detection step of amplified product by agarose gel electrophoresis.

The detection limit of the developed LAMP assay in this study was 10 pg/µL which is a good low limit accepted for laboratory diagnostic assay. There was no cross-reaction with other *burkholderia* species and other gram-

negative bacteria. These results indicated that the LAMP assay was highly specific for *B. pseudomallei*. The high specificity and sensitivity, the short time for analysis, the cost effectiveness, non-requirement of expensive equipment are the key advantages of LAMP assay.

In conclusion LAMP technique represents a reliable alternative molecular isothermal amplification technique for the rapid diagnosis of melioidosis by the detection of *B. pseudomallei* with the advantage of field use and is fast, sensitive and specific in different types of samples. The results of this study give evidence that this LAMP technique can be used as point of care laboratory diagnostic test and can be used also as molecular amplification technique for rapid detection of *B. pseudomallei.*

Acknowledgement

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List of Tables and Figures

Table 1 Primers designed for amplification of *B. pseudomallei*.

Primer Name	Length	Sequence (5' – 3')
F3	16	gggATgCCTCgCCAAC
B3	19	gCgCTATgCggAAgTCATC
FIP	45	TATTCggCgTggAggCgAAAAATTTTCgACATACTCCgATgCggC
BIP	45	CgTTgTgCAgACCCACCgATTTTTTTgAgAACTCggCgAgTTgg
LF	19	TCAggTgCTggCTCATCAA
LB	20	TgCAgACgACgTCTCCACAT

Table 2 Diagnostic performance of LAMP assay to detect *Burkholderia pseudomallei* in all 91 clinical samples

Strains	Total no. of samples	Number of samples detected by LAMP (culture method)		Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
		Positive	Negative				
B. pseudomallei	73	73 (73)	0 (0)	100	100	100	100
B. mallei	3	0 (0)	3 (3)	100	100	100	100
B. thailandensis	10	0 (0)	10 (10)	100	100	100	100
Other bacteria strains	5	0(0)	5(5)	100	100	100	100

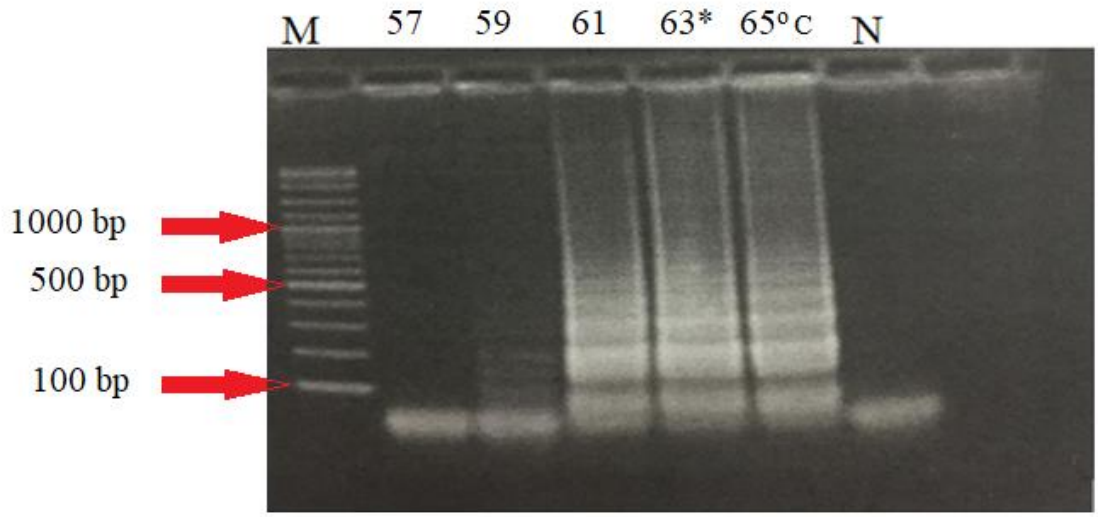

Figure 1 Optimal reaction temperature of LAMP assay. M: DNA Marker, N: Negative control. (* indicates the optimal temperature)

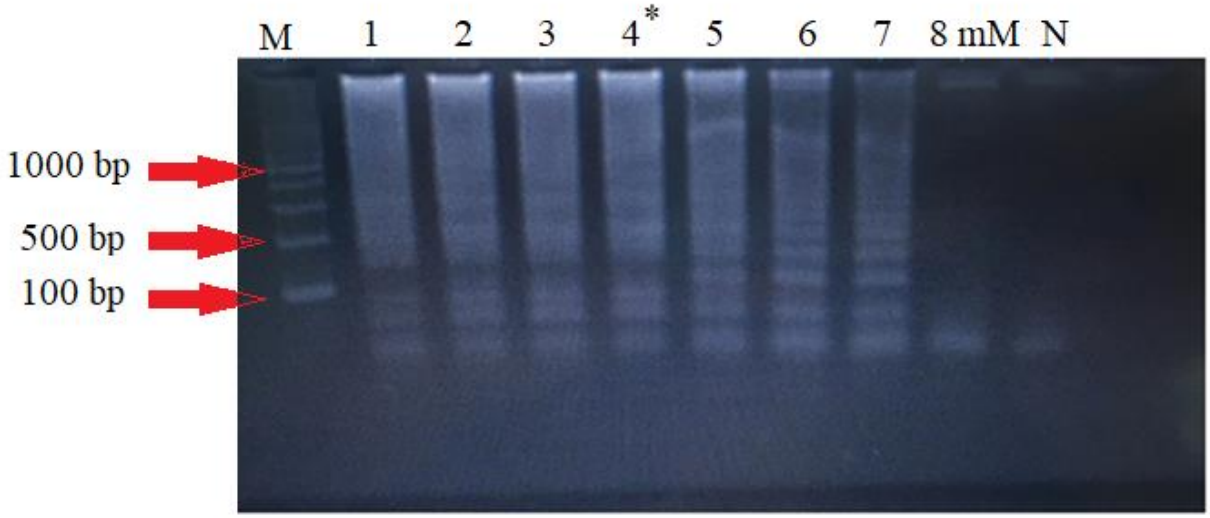

Figure 2 Optimal Mg^{2+} concentration of LAMP assay. M: DNA Marker, N: Negative control. (* indicates as optimal concentration.)

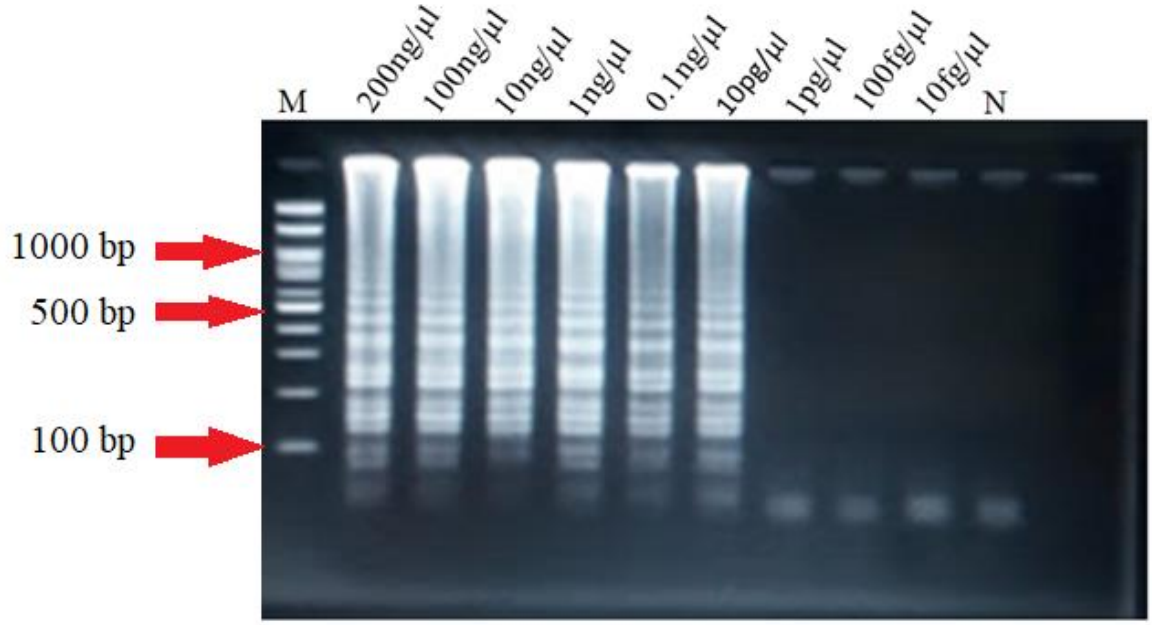

Figure 3 Detection limit of LAMP assay for the detection of *B. pseudomallei* M: DNA marker, N: Negative control

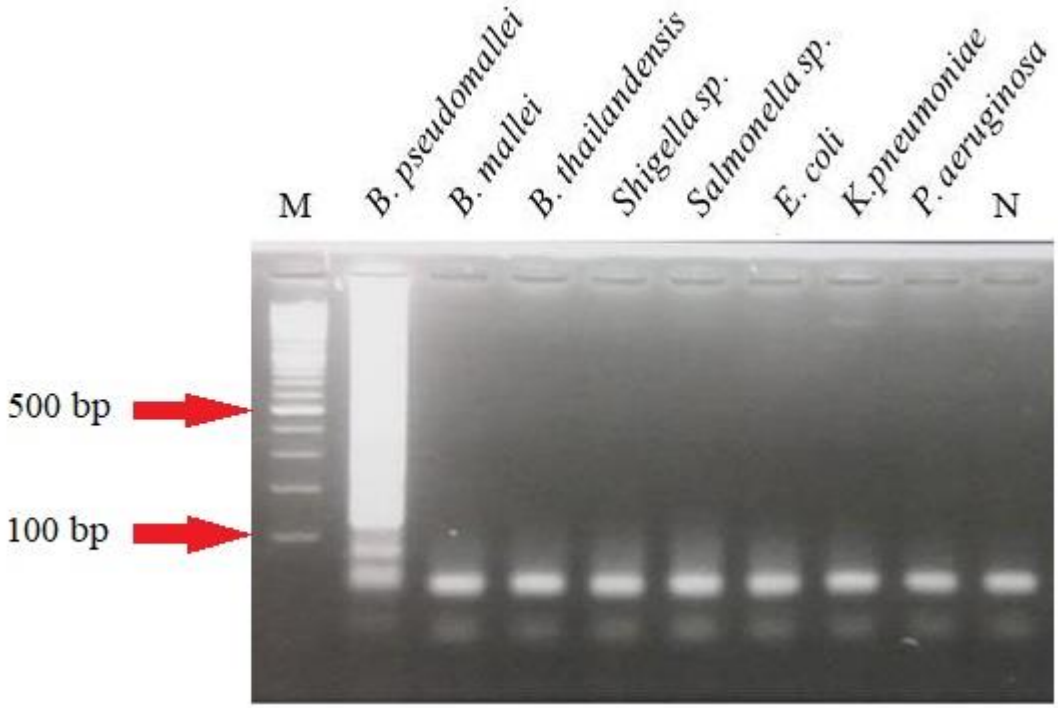

Figure 4 Specificity of LAMP assay for *B. pseudomallei.* M: DNA marker, N: negative control