นิพนธ์ต้นฉบับ ▪ Original Article

การปรับปรุงวิธีการตรวจแบบทำให้เกิดสีของวิธี Loop Mediated Isothermal Amplification เพื่อตรวจหาเชื้อมัยโคแบคทีเรียม ทิวเบอร์คิวโลสิส

จิตตนันท์ ศรีสุทัศน์[1], อรุณนี สังกา[2], วิเศษ นามวาท[3]

[1]*นักศึกษาปริญญาโทสาขาวิชาจุลชีววิทยาทางการแพทย์ ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์มหาวิทยาลัยขอนแก่น*

[2]*ภาควิชาจุลชีววิทยาคลินิก คณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น*

[3]*ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น*

Optimization of Colorimetric Assay of Loop Mediated Isothermal Amplification for Detections of *Mycobacterium tuberculosis*

Jittanun Srisutush[1], Arunnee Sangka[2], Wises Namwat[3]

[1]*Student, Master of Medical Microbiology, Department of Microbiology, Faculty of Medicine, Khon Kaen University*

[2]*Assistant Professor, Department of Clinical Microbiology, Faculty of Associated Medical Sciences, Khon Kaen University*

[3]*Assistant Professor, Department of Microbiology, Faculty of Medicine, Khon Kaen University*

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หลักการและวัตถุประสงค์: วัณโรคมีสาเหตุจากเชื้อมัยโคแบคทีเรียม ทิวเบอร์คิวโลสิส (*Mycobacterium tuberculosis*) เป็นปัญหาหลักทางสาธารณสุขของไทยและทั่วโลก นอกจากนี้ยังเป็นสาเหตุการตายอันดับที่สองของการเสียชีวิตจากโรคติดเชื้อทั่วโลก การพัฒนาการตรวจวินิจฉัยวัณโรคเป็นปัจจัยสำคัญอย่างหนึ่งในการควบคุมวัณโรค การศึกษานี้มีวัตถุประสงค์เพื่อปรับการตรวจวัดด้วยสีให้เหมาะสมที่สุดในขั้นตอนการตรวจวัดหลังการเพิ่มปริมาณ DNA ด้วยเทคนิค Loop-mediated isothermal amplification (LAMP) ซึ่งเป็นวิธีที่สะดวกและรวดเร็วในการตรวจวินิจฉัย

วิธีการศึกษา: ได้ใช้เทคนิค LAMP เพื่อใช้ในการตรวจหาดีเอ็นเอของเชื้อ มัยโคแบคทีเรียม ทิวเบอร์คิวโลสิส ในขั้นตอนการตรวจหาผลผลิตของการเพิ่มจำนวนดีเอ็นเอ มีการใช้ Hydroxy naphthol blue (HNB), Sybergreen (SYBR) และ gel electrophoresis มีการปรับปริมาณและวิธีการใช้ HNB เปรียบเทียบผลจากการใช้ SYBR และ gel electrophoresis

ผลการศึกษา: การใช้ HNB ในการเติมล่วงหน้าก่อนปฏิกิริยาเพิ่มจำนวนดีเอ็นเอ ความเข้มข้นที่เหมาะสมคือ 5 mM โดยผลบวกจะมีการเปลี่ยนสีจากสีม่วงเป็นสีฟ้าและไม่รบกวนปฏิกิริยา การตรวจหาปริมาณ DNA ของเชื้อ MTB ในระดับต่ำสุด ระหว่างการใช้ HNB, SYBR และ gel electrophoresis คือ 10, 100 และ 10 pg ตามลำดับ

สรุป: การตรวจแบบทำให้เกิดสีของวิธี LAMP โดยใช้ HNB ได้

Background and objectives: Tuberculosis is an airborne disease caused by *Mycobacterium tuberculosis* (MTB). It is a major public health problem of Thailand and worldwide, ranking as the second leading cause of death from worldwide infectious diseases. Diagnosis development is one of key factors to control it. The objective of this study was to optimize the colorimetric method of Loop-mediated isothermal amplification (LAMP), which is a new promising method because DNA is synthesized at a single temperature in relatively short period.

Methodology: The LAMP was set-up to detect the genomic DNA of MTB. The amplified product was detected by using Hydroxy naphthol blue (HNB), Sybergreen (SYBR) and gel electrophoresis. The amount of HNB was optimized for detection compare to the results of using SYBR and gel electrophoreses.

Results: Pre-addition of 5 mM HNB to the LAMP reaction solution showed observe-able blue-sky color of positive but did not interfere amplification efficiency. The minimum detection limit between using HNB, SYBR and gel electrophoresis were 10, 100 and 10 pg of MTB genomic DNA, respectively.

*Corresponding author : Wises Namwat, Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen province, Thailand. E-mail: wisnam@kku.ac.th

รับการปรับให้เหมาะสมสำหรับการตรวจหาเชื้อMTB ความเข้มข้นที่เหมาะสมของ HNB คือ 5mM และตรวจหาปริมาณ DNAของเชื้อ MTB ในระดับต่ำสุดที่ 10 pg ซึ่งคล้ายกับ gel electrophoresis

คำสำคัญ: วัณโรค

Conclusion: The colorimetric LAMP method using HNB was successfully optimized for detection of MTB. The optimal of concentration of HNB was 5mM and the minimum detection limit was 10 pg of MTB genomic DNA which is similar to those of gel electrophoresis.

Keywords: LAMP; Tuberculosis; HNB; Loop-Mediated Isothermal Amplification; Hydroxy naphthol blue

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Introduction

Tuberculosis (TB) remains a major public health problem worldwide. Approximately, one third of the world populations are infected with TB. It is the first cause of death due to a single infectious agent in adults[1]. It is estimated that nearly 1 billion people will be newly infected with TB between 2000 and 2020, furthermore, two hundred million people will develop disease and 35 million will die from TB within this period1. Recently, the incidence of human mycobacterial infections due to species other than *M. tuberculosis* has increased worldwide.

Many methods have been developing for the diagnosis of TB. In previous study, the conventional methodology, which includes specimen treatment, microscopic examination for acid-fast bacilli, isolation with the use of solid and/or liquid culture, and the classic differentiation with biochemical tests is slow and takes several weeks. Over the last few years, new molecular methods have been introduced, including PCR-Restriction Fragment Length Polymorphism, real-time PCR, DNA sequencing, and DNA strip assays as mycobacterial diagnostic tools, leading to considerable improvement of both speed and accuracy of identification. Loop-mediated isothermal amplification (LAMP), is an auto cycling and strand displacement DNA synthesis method involving the use of the large fragment of Bst DNA polymerase and a set of six specially designed primers[2].

LAMP assays have been used for detection of several types of infections, including ocular tuberculosis infectioin[3]. Because LAMP reactions can be performed at a constant temperature ranging from 60°C to 65°C, the amplification specificity is extremely high because the LAMP reaction requires a set of six oligonucleotide primers that recognize six distinct regions on the target DNA, the detection limit of LAMP is expected to be equal to or higher than that of PCR, the detection time is shorter and visualization of DNA products on gel electrophoresis is not required for assessing successful DNA amplification because a positive LAMP reaction causes the solution to become cloudy due to the formation of the magnesium pyrophosphate byproduct[2]. The turbidity of the solution has a high correlation with the amount of DNA synthesized, and a real-time turbid meter for the LAMP reaction was developed for quantifying initial template DNA[4].

Several studies have reported the use of the LAMP method for detecting various pathogens[2]. However, many of these studies used an expensive real-time turbid meter or a real-time PCR system for the reaction confirmation. The use of expensive equipment decreases the versatility of LAMP and greatly limits the wide use of this procedure, especially in developing countries. Detection of turbidity by the naked eye is the simplest and most cost efficient method for judging a positive or negative LAMP reaction, although this method requires some skill for assessing the result. For better visibility of the reaction result, a DNA intercalating dye such as SYBR green, Picogreen, or propidium iodide is added to the solution after the reaction is completed. When the LAMP reaction is positive, a color change is observed under ambient light. However, as in the case of analysis in gel electrophoresis, the colorimetric assay using the intercalating dye is associated with an increased risk of contamination of other subsequent LAMP reaction solutions because the assay requires opening of the tubes. To avoid such contamination, separate rooms should be used for LAMP setup and analysis.

The LAMP reaction results in large amounts of pyrophosphate ion byproduct; these ions react with Mg^{2+} ions to form the insoluble product magnesium pyrophosphate. Since Mg^{2+} ion concentration decreases as the LAMP reaction progresses, the LAMP reaction can be quantified by measuring the Mg^{2+} ion concentration in the reaction solution[2]. Hydroxy

naphthol blue (HNB) was reported to be useful as a colorimetric indicator for the titration of Ca^{2+} ions and Mg^{2+} ions[2]. Therefore, HNB could be a novel indicator for the LAMP reaction by monitoring the change in the Mg^{2+} ion concentration since the large fragment of *Bst* DNA polymerase synthesizes DNA under alkaline conditions[2]. This color change is induced by the chelation of Mg^{2+} ions by dNTPs[2]. HNB was used for a new colorimetric assay of the LAMP reaction. A positive reaction is indicated by a color change from violet to sky blue[2]. Moreover HNB was not required opening the reaction tube this reduces the risk of cross-contamination.

In this study, we aimed to optimize a simpler colorimetric assay for the detection of the LAMP reaction by using another metal ion indicator namely HNB. This colorimetric assay is superior to the existing colorimetric assays for LAMP with regard to reducing contamination risks, and is helpful in high-throughput DNA and RNA detection.

Methodology

Primer validation

LAMP it is characterized by the use of 6 different primers specifically designed to recognize 6 distinct regions on the target gene and the reaction process proceeds at a constant temperature using strand displacement reaction. A set of primers specific to *mpb* 64 gene were obtained from the previous study [3] (Table 1). The mycobacterial DNA sequences were obtained from GenBank (https://www.ncbi.nlm.nih.gov). The primers were mapped in the mycobacterial sequence by Bio-Edit program. Primer-dimer formation, hair-pin structure were analyzed by OligoAnalyzer (https://sg.idtdna.com/calc/analyzer) (Table 1).

Optimization of LAMP condition

The condition of LAMP was optimized by using an Applied Bio systems Quant Studio 6 Flex Real-Time PCR System. LAMP reactions were performed in a volume of 25 µl consisting of 1 µl of reference genomic DNA, 2.5 µl of target-specific LAMP primer mix (2 µM each of F3 and B3 primers, 8 µM each of Loop-F and Loop-B primers, 16 µM each of FIP and BIP primers), 12.5 µl of Lava LAMP™ DNA Master Mix, 8 µl Nuclease-free H_2O and 1 µl of Green Fluorescent Dye or HNB. Lava LAMP™ DNA Master Mix with Dye (LCG-30067-1) was purchased from Lucigen (Middleton, Wisconsin, USA) and HNB were purchased from Dojindo (Kamimashiki, Kumamoto, Japan). Stock solution of HNB was prepared by dissolving in distilled water at 20 mM. HNB concentration in the reaction was varied from 1 to 6 mm. Syber Green (SYBR) included in the master mix kit was used 1 ul per reaction. The LAMP reaction was performed at 70 °C for 40 min. Reaction mixtures without DNA and with reference strain DNA was prepared to serve as negative controls and positive controls, respectively. The complete reaction tube using HNB and SYB was visualized on a light box and UV box, respectively. The amplicon was confirmed by gel electrophoresis and staining with ethidium bromide.

Lower detection limit of LAMP

The lower detection limit of LAMP was determined by using ten-fold serial dilution chromosomal DNA of *M. tuberculosis* H37Rv varied form 10 ng to 10 fg per LAMP reaction.

This study was approved by Khon Kaen University Ethics Committee for Human Research, Khon Kaen University, Thailand. (Reference No. HE611145).

Results

Pre-addition of 5 mM HNB to the LAMP reaction solution showed observe-able blue-sky color of positive but did not inhibit amplification efficiency of the large fragment of Bst DNA polymerase.

Table 1 Primer used for *M. tuberculosis* LAMP assay targeting the mpb 64 gene.

Primer name	Primer sequence (5'-3')
MTB-F3	CCCCCGGGTTGAAGAAGA
MTB-B3	GGCCTATCGCAAGCCAAT
MTB-FIP	GTATCGATAGCGCCGAATGCCGT - TTTCGTTCGTGACTGCGAAGT
MTB-BIP	TGCTTGCTCAGTTCACCTTGCAT - TTTCACCTATGACACGCTGTGG
MTB-FLP	GCTTGGACCCGGTGAATTATCAGA
MTB-BLP	AGCGGATCGGTGTCAGCCT

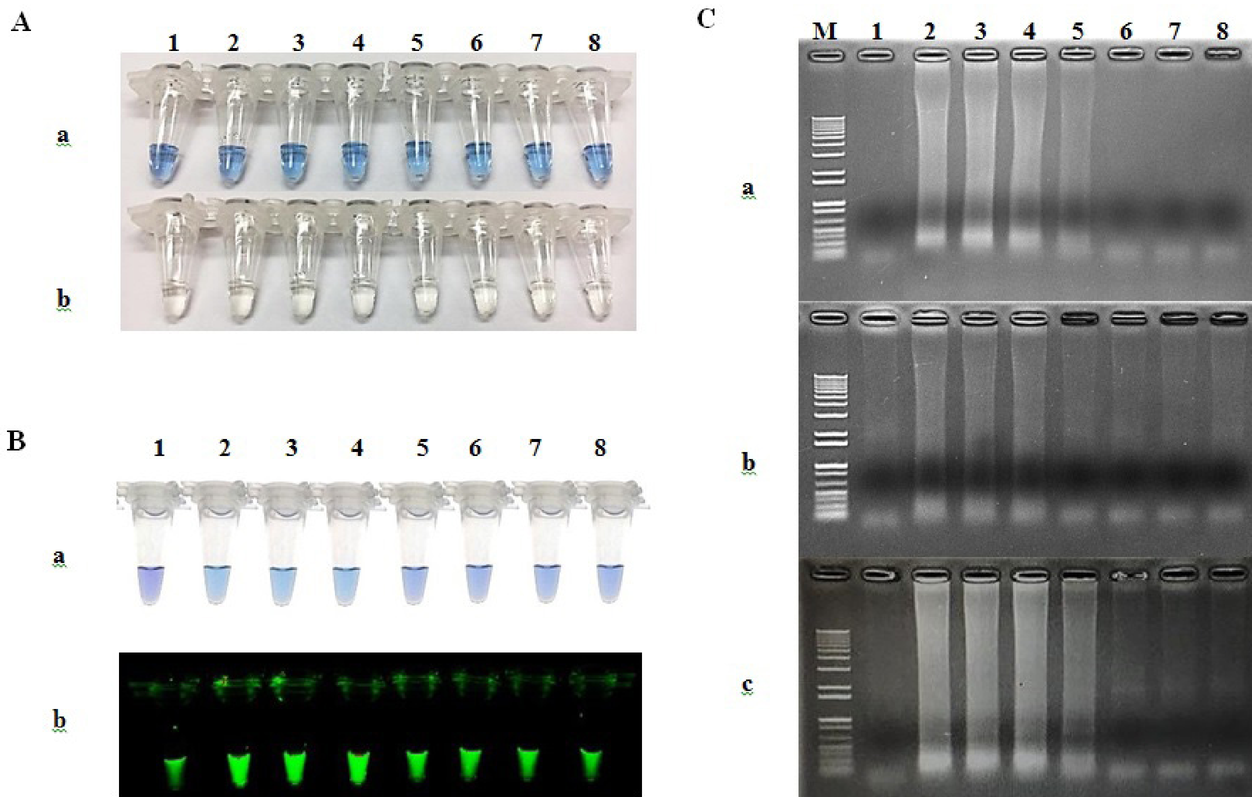

Figure 1 Comparative lower detection limit of LAMP assay using different dyes for the detection of serially diluted DNA. Tube or lane 1 to 8 were negative, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg and 10 fg of DNA, respectively. (A) Color of reaction mixture before start LAMP reaction. (a) HNB and (b) SYBR green. (B) Color of reaction mixture after running LAMP reaction. (a) HNB: visualization under visible white light box, Conversion from violet to sky-blue indicated a positive reaction. (b) SYBR green: visualization under UV light box. Bright fluorescence indicates a positive reaction. (C) Gel electrophoresis. (a) HNB, (b) SYBR green (c) without dye.

The performances of LAMP assays using HNB, SYBR green, and without dye were compared with regard to the detection of ten-fold serial dilution from 10 ng to 10 fg per LAMP reaction (Figure 1). The color change of HNB from violet to sky-blue indicated a positive reaction. The lower detection limit of the LAMP assay using HNB was 100 pg (Figure 1B, tube a4), which was similar to that of the assay using SYBR green (Figure 1B, tube b4). Gel electrophoresis of the amplified product from the reaction with HNB, SYBR and without dye show in Figure 1C. A smear band indicates positive result of the amplification. The lower detection limit was found at 10 pg, 100 pg and 10 pg, respectively.

Discussion

The colorimetric assay for LAMP carried out by adding HNB to the reaction solution should be optimized. Although high concentration of HNB showed more contrast between positive and negative color, but high concentration of the color could interfere efficiency of the amplification which might cause false negative result in case of low concentration of the DNA template in the reaction.

Lower detection limit of the LAMP using HNB was 100 pg which is equivalent to 20,000 copies of *M. tuberculosis* chromosome. It was 10 times higher than that of detection by using gel electrophoresis. Whereas sensitivity of the method was firstly report at 6 copies of the viral chromosome[5]. The lower detection limit of this study was relatively high and should be improved for further development.

This colorimetric LAMP assay using HNB has some benefits compared with other nucleic acid amplification techniques including easy operation, no need for special equipment, superior sensitivity and speed, low contamination risk, and suitability for DNA detection. It would be helpful for tuberculosis diagnosis.

Conclusions

The colorimetric LAMP method using 5mM HNB could be used for tuberculosis diagnosis. The minimum detection limit was 100 pg of genomic DNA of MTB.

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