Development of Loop-Mediated Isothermal Amplification Assay for Rapid Detection of bla_{CTX-M1} Gene in *Escherichia coli* and *Klebsiella pneumoniae*

การพัฒนาวิธี loop-mediated isothermal amplification สำหรับการตรวจสอบยีน bla_{CTX-M1} ในเชื้อ *Escherichia coli* และ *Klebsiella pneumoniae* อย่างรวดเร็ว

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ABSTRACT

Extended-spectrum β-lactamases (ESBLs) have been rapidly spreaded in many regions of the world and become major problem in hospital and community. Thus, rapid and accurate detection method of ESBLs is essential. The objective of this study was to develop loop-mediated isothermal amplification (LAMP) method for rapid detection of bla_{CTX-M1} gene in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae.* The efficacy and specificity of LAMP method were compared with those of conventional PCR method. The results showed that LAMP could detect bla_{CTX-M1} gene under optimal condition at 63 ºC within 60 min, while conventional PCR required approximately 2 hours. In addition, both LAMP and conventional PCR showed 100 % specificity in detection of bla_{CTX-M1} gene from bacterial isolates that the results showed found in 11 of 35 isolates (31.43%) . These results indicated that LAMP method is a rapid, accurate and simple method for the detection of bla_{CTX-M1} gene.

บทคัดย่อ

เอนไซม์เบต้าแลคแตมเมสชนิดออกฤทธิ์กว้าง ได้มีการแพร่กระจายอย่างรวดเร็วในหลายพื้นที่ของโลกและกลายเป็นปัญหาใหญ่ทั้งในโรงพยาบาลและแหล่งชุมชน ดังนั้นการตรวจสอบที่รวดเร็วและถูกต้องในการตรวจหาเอนไซม์เบต้าแลคแตมเมสชนิดออกฤทธิ์กว้างนี้จึงเป็นสิ่งจำเป็นอย่างยิ่ง การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาวิธี loop-mediated isothermal amplification (LAMP) สำหรับการตรวจสอบยีน bla_{CTX-M1} ในเชื้อ *Escherichia coli* และ *Klebsiella pneumoniae* ที่แยกได้จากผู้ป่วย จากการเปรียบเทียบประสิทธิภาพและความจำเพาะกับวิธีพีซีอาร์ พบว่าวิธี LAMP สามารถตรวจสอบยีน bla_{CTX-M1} ภายใต้สภาวะที่เหมาะสม คือ 63 องศาเซลเซียล เป็นเวลา 60 นาที ขณะที่วิธีพีซีอาร์ปฏิกิริยาใช้ระยะเวลาในการตรวจสอบประมาณ 2 ชั่วโมง นอกจากนี้ยังพบว่าทั้งวิธีพีซีอาร์และวิธี LAMP มีความจำเพาะ 100% ในการตรวจสอบหายีน bla_{CTX-M1} ซึ่งจากผลการทดลองพบ 11 เชื้อ จาก 35 เชื้อ (31.43%) แสดงให้เห็นว่าวิธี LAMP มีความรวดเร็ว ถูกต้อง และง่ายต่อการตรวจสอบยีน bla_{CTX-M1}

Key Words: Extended-spectrum β-lactamases (ESBLs), PCR method, LAMP method

คำสำคัญ: เอนไซม์เบต้าแลคแตมเมสชนิดออกฤทธิ์กว้าง วิธีพีซีอาร์ วิธี LAMP

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Introduction

Extended-spectrumβ-lactamases (ESBLs) are bacterial enzymes that produced by Gram-negative bacteria including *Enterobacteriaceae*. ESBLs are able to break down most of β-lactam antibiotics such as penicillins, cephalosporins or extended-spectrum cephalosporin (first, second and third-generation) and monobactams (Pitout JD, Laupland KB, 2008) . ESBLs-producing genes are usually located on large plasmids that spread quickly from strain to strain and between bacterial species worldwide (Pitout JD, 2010) . Kiratisin P and colleagues reported that a total of 87.3 % of bacterial isolates carried several *bla* genes (Kiratisin P, 2008). Thus, ESBLs are an important cause of antimicrobial-resistance. The ESBLs detection method can be classified into 2 groups; phenotypic method and genotypic method. The phenotypic methods are based on non – molecular technique. These methods detect the ability of ESBLs to hydrolyze the 3rd generation cephalosporins together with β-lactamase inhibitor and can differentiate between ESBLs and non – ESBLs. The genotypic methods are based on molecular technique that can be identified the specific gene of ESBLs.

Cefotaximase (CTX-M) is a new family of plasmid-mediated ESBLs that was first isolated in Munich (Pitout JD, 2010) . At present, the new phylogenetic tree of CTX-M family based on amino acid sequence can be divided into 7 groups consisting of CTX-M2, CTX-M3, CTX-M8, CTX-M14, CTX-M25, CTX-M45 and CTX-M64 (Zhao and Hu, 2012). At present, the groups of CTX-M enzymes play an important role in the incidences of ESBLs outbreak in the world because CTX-M-coding sequences were located in mobile element (Bonnet, 2004) . In Thailand, there is a report showing that CTX-M is the most prevalent in ESBL-producing *E. coli* and *K. pneumoniae* with 99.6% and 99.2% respectively (Kiratisin P, 2008).

In this study, we focus on developing a new genotypic method to detect bla_{CTX-M1} gene. Generally, PCR is the genotypic method most widely used for detection of ESBLs-coding sequences. However, PCR method is expensive and requires special equipment such as PCR machine and gel documentation.

Loop-mediated isothermal amplification (LAMP) is a novel amplification technique that amplified DNA under isothermal condition in one step for less than 1 hour with *Bst* DNA polymerase strand displacement activity and 4-6 specific primers that recognized 6-8 regions on the DNA template (Notomi T, 2000) . LAMP method showed high specificity, sensitivity and rapidity for detection of bacterial infections such as *Mycoplasma pneumoniae* (Saito R, 2005) and *Burkholderia pseudomallei* (Chantratita N et al., 2007) . Moreover, LAMP method was also developed for rapid diagnosis of virus infection such as Herpes Simplex Virus (Enomoto Y et al., 2005), Dengue viruses (Teoh BT et al., 2013) , Human Herpesvirus 6 (Ihira M et al., 2004) and Varicella–Zoster Virus (Okamoto S, 2004).

Objective

To develop loop-mediated isothermal amplification (LAMP) method for rapid detection of bla_{CTX-M1} gene in *Escherichia coli* and *Klebsiella pneumoniae*.

Methodology

Bacterial isolates

A total of 35 ESBL-producing Enterobacteriaceae isolates including 27 strains of *E. coli* and 8 strains of *K. pneumoniae* were investigated in this study. These bacterial strains were isolated from various specimens of patients who received treatment at the Ramathibodi hospital between 2005 and 2006. *Salmonella* Typhi was used as the positive strain of bla_{CTX-M1} gene.

Double-disk synergy method

The production of ESBL was confirmed by double disk synergy test (DDST). Single colony of each bacterial were cultured in 5 ml of MH broth and incubated at 37ºC for 5 hours with continuous shaking at 150 rpm until turbidity of bacterial suspension reach 0.5 McFarland and swab on MH agar. Both cefotaxime (30 µg) and ceftazidime (30 µg) alone and combination of amoxicillin/clavulanic acid (20 µg/10 µg) were used. These plates were incubated at 37 ºC for 18 hr. Enhancement of the inhibition zone between the disk containing clavulanic acid and cefotaxime or ceftazidime indicated the presence of ESBL production.

Preparation of DNA template

A single colony of each isolate was grown in 5 ml LB broth containing 100 µg/ml ampicillin (USB Corporation, Ohio, USA) and incubated at 37 ºC for overnight with continuous shaking at 150 rpm. All DNA templates used in this study were prepared by alkaline lysis method (Sambrook and Russell, 2001) with modification and dissolved in sterile deionized water.

Primer design of the bla_{CTX-M1} gene for PCR and LAMP method

PCR and LAMP method were developed for bla_{CTX-M1} of ESBLs producing gene. The primers for PCR amplification were forward primer (5'-CGGTGC TGAAGAAAAGG-3') and reverse primer (5'-TGTCACC CAATGCTTTACC-3') . The amplicon size was approximately 369 bp. A set of 4 LAMP primers were designed using the PrimerExplorer V4 program (PrimerExplorer, Eiken Chemical Co. Ltd.) as shown in Table1.

Table 1 PCR and LAMP primers for bla_{CTX-M1} gene used in this study.

Method	Primer	Sequence (5' to 3')
PCR	Forward	CGGTGC TGAAGAAAAGG
	Reverse	TGTCACC CAATGCTTTACC
LAMP	F3	GCAATGGCGCAAACTCTG
	B3	TTGGTGGTGCCATAGTCAC
	FIP	TCATCCATGTCACCAGCTGCG
		AATCTGACGCTGGGTAAAGC
	BIP	AGGCAATACCACCGGTGCAGC
		GGTTTTATCCCCCACAACC

Polymerase chain reaction (PCR) method

The reaction was performed with a final volume 25 µl containing 0.4 µM of Forward and Reverse primers, 4U of Q5 high-Fidelity DNA polymerase with 1x Q5 reaction buffer (New England Biolab, Ipswich, MA, USA, 0.2 mM each of deoxynucleotide triphosphate (dNTPs) (Roche, Mannheim, Germany) , template DNA (100 ng) and sterile deionized water to adjust volume. The reaction consisting of pre-heat at 94 ºC for 1 min, denaturation at 94ºC for 1 min, annealing at 62ºC for 30 sec, extension at 72ºC for 1 min and final extension at 72ºC for 5 min, with repeated 30 cycle in a GeneAmp® PCR system 2700 (Applied Biosystems, California, USA.). The PCR product were separate by 1.5% agarose gel electrophoresis and stained with

ethidium bromine and visualized by UV light in gel documentation system.

Loop - mediated isothermal amplification (LAMP) method

The LAMP reaction was performed in a total 25 µl reaction mixture containing 4U *Bst* DNA polymerase with 1X thermopol buffer (New England Biolab, Ipswich, Massachusetts, USA), 200 µM each dNTPs (Roche, Mannheim, Germany), 1 M betaine (Sigma-Aldrich, Munich, Germany), 6 mM $MgSO_4$, 0.8 µM each FIP and BIP, 0.2 µM each F3 and B3, 1 µl of DNA template (100 ng) and adjust volume with sterile deionize water. The reaction was running at 63ºC for 60 min, followed by heating at 80ºC for 2 min to stop the reaction. Sterile deionized water was used as negative controls. The LAMP products were analyzed by electrophoresis on 1.5% agarose gel and visualized by UV light. The LAMP product was also detected by naked eyes using SYBR green I. After amplification, the tubes were inspected of a color change by addition of 1.0 µl of 1:10 diluted SYBR Green I to the LAMP products. Restriction endonuclease digestions were used to confirm LAMP product by digest with *Age*I (New England Biolab, Ipswich, Massachusetts, USA) and *Pvu*II (New England Biolab, Ipswich, Massachusetts, USA). The digestion mixture contained 1xbuffer, 5U restriction endonuclease and nuclease free water in 20 µl reaction volume. The reaction mixtures were incubated at 37ºC for overnight and incubated at 65ºC for 20 min to stop enzyme activity. The DNA fragments were separated by electrophoresis on 3% agarose gel electrophoresis.

Result

Double disk synergy test (DDST) was performed to confirm the production of ESBLs in 35 clinical isolates .The result of DDST showed that all isolates are positive for ESBLs production (data not shown). The PCR amplicon size of bla_{CTX-M1} gene product, approximately 369 bp, was found in 11 of 35 isolates (31.43%) including isolate number 3, 4, 5, 7, 11, 13, 21, 22, 24, 27 and 34 (Figure 1). To confirm the identity of PCR products, the nucleotide sequence of each sample was determined and compared with the β-lactamase genes in the GenBlank database. The results showed high percentage of sequence homology with bla_{CTX-M} group of β-lactamase, approximately 99% and the sequence of PCR product of each isolates was 99% similarity to each other.

Moreover, the 35 clinical isolated samples showed the consistent results after tested with LAMP and PCR methods. All the positive LAMP reactions produced characteristic ladder-like patterns on an agarose gel (Figure 2). The detection of LAMP product by naked eyes visualization with the addition of SYBR green I showed the positive result of observing the color change to green color of the amplification products (Figure 3A). All amplified products were digested with *Age*I and *Pvu*II, in order to confirm the results. The prediction sites were 110, 65, 15 and 11 bp when digested with *AgeI* and 125, 108 and 50 bp when digested with *Pvu*II (Figure 3B). The sizes of the fragments were in good agreement with the predicted size from the DNA structure 15 of bla_{CTX-M1} gene sequence.

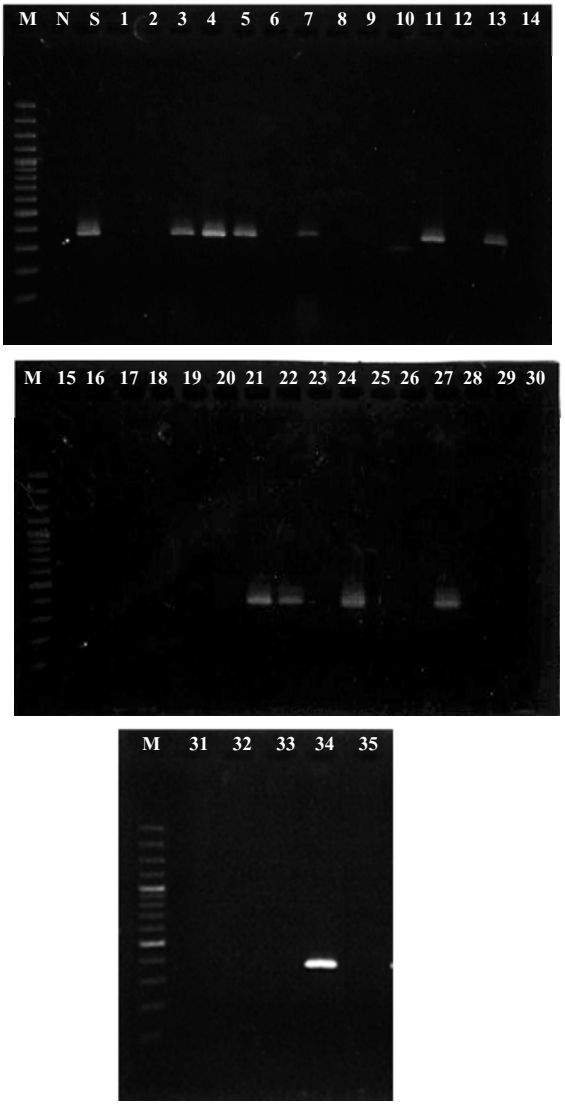

Figure 1 PCR amplification results of bla_{CTX-M1} gene of isolate numbers 1-35. Lane M, 100 bp DNA ladder plus, lane N, negative control without DNA template, lane S, positive control with plasmid of *S.* Typhi as a DNA template, lanes 1-35, isolate 1-35, respectively.

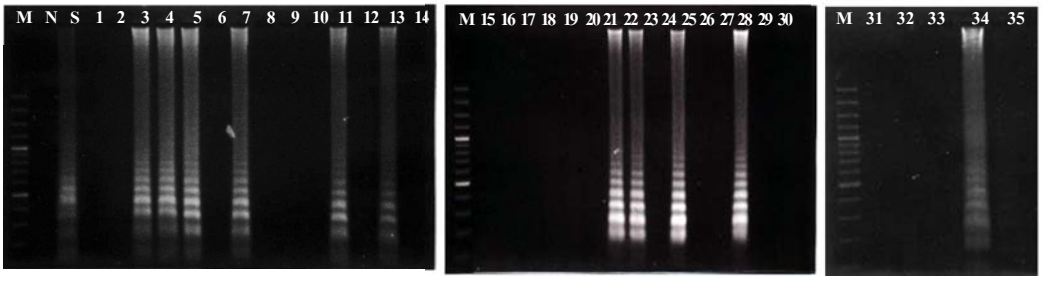

Figure 2 The DNA gel electrophoresis pattern of bla_{CTX-M1} gene after detected with LAMP method. Lane M, 100 bp plus DNA ladder; lane N, negative control (without template); lane S, positive control (plasmid of *S.* Typhi); lanes 1-35, test samples.

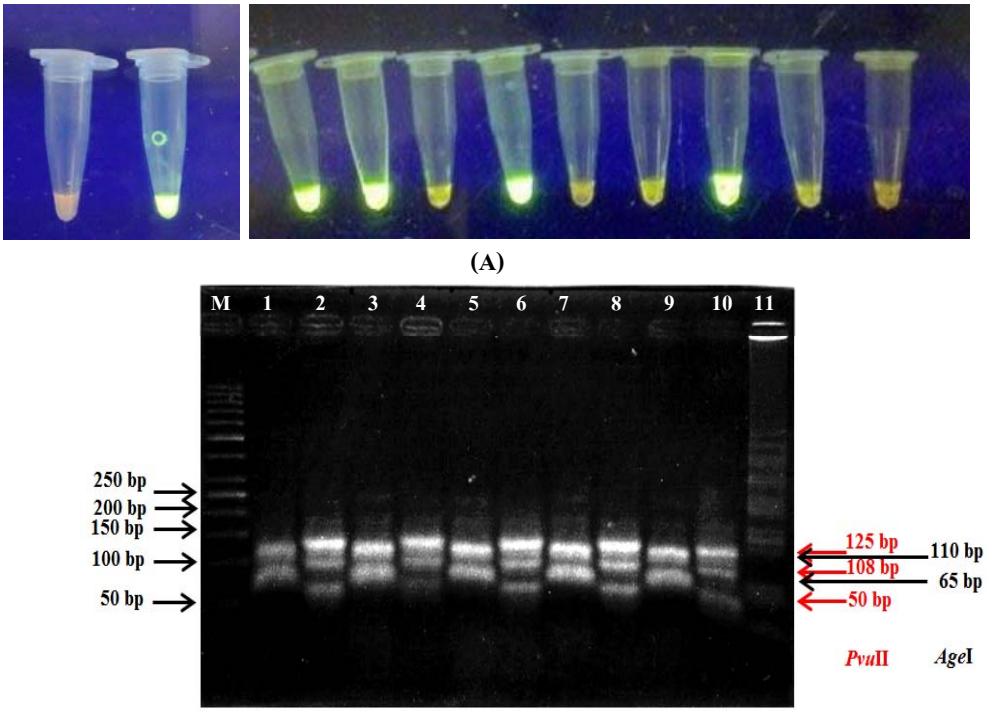

Figure 3 Detection of LAMP products under UV light at 365 nm after mixed with SYBR Green I **(A):** tube 1; the negative result (orange), tube 2; positive result (luminescence green); tube 3-11, test samples. The digestion patterns of LAMP products after digested with *Age*I (lane 1, 3, 5, 7, and 9) and *Pvu*II (lane 2, 4, 6, 8, and 10). Lane M, 50 bp DNA ladder **(B)**.

Discussion and conclusion

Polymerase chain reaction (PCR) method is the most widely genotypic method used for detecting the specific gene of ESBLs (Rupp ME, 2003, Krishnamurthy V, 2013) . The advantages of PCR method are high sensitivity and specificity. However, the disadvantages of PCR assay are more instruments requirement and time consumable than the LAMP method (Lim KT et al., 2013).

In 2000 Notomi et al., was developed loop-mediated isothermal amplification (LAMP) method for DNA amplification with high efficiency and rapidity under isothermal conditions (Notomi T, 2000). Many study have been reported the helpfulness of the LAMP method for detection of pathogenic microorganisms such as *Staphylococcus aureus* (Lim KT et al., 2013) , *Trypanosoma brucei rhodesiense* (Njiru ZK et al., 2008), *Haemphilus parasuis* (Yang W et al., 2010) and *E.coli* O157:H7 (Sasitharan D et al., 2013).

In this study, we developed LAMP method for rapid detection of bla_{CTX-M1} gene from 35 clinical isolates. The screening result showed 100 % specificity between PCR and LAMP methods. The LAMP amplicon showed specific amplification product generated many ladder-like pattern bands on agarose gel. This ladder-like pattern is characteristic of the LAMP amplification and indicates that stem-loop DNA with invert repeats of the target sequence was produced.

Although LAMP and PCR showed the same result the reaction time of LAMP method is shorter

than PCR method because of the isothermal without thermal changes between steps. In addition, the detection methods to detect LAMP product by naked-eye with SYBR I dose not require gel documentation system that indicating LAMP method is a more rapid method.

In conclusion, the result of LAMP method is comparable to PCR method in detection of ESBLs – producing gene. However, LAMP assay is rapid, accurate and dose not requires special equipment for the detection of ESBLs – producing gene.

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