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Development of Chlamydia trachomatis detection by loop-mediated isothermal amplification

[Ilada Choopara, Naraporn Somboonna]

Abstract **- An obligate intracellular human pathogen *Chlamydia trachomatis* is the most common bacterial cause of sexually transmitted diseases in females and males worldwide. The diseases range from urethritis, cervicitic, pelvic inflammatory disease, ectopic pregnancy, infertility to trachoma (eye infection) in newborns from infected mothers. Currently, the standard method for *C. trachomatis* detection in laboratory is polymerase chain reaction (PCR) of the gene encoded for a major outer membrane protein (*ompA*) followed by electrophoresis. This process requires several hours (3.5 hrs. PCR and sequencing), handling of carcinogenic ethidiumbromide during gel electrophoresis, and is expensive. Thermocycling machine and electrophoresis apparatus are high-priced. Thus, the current research developed *C. trachomatis* detection by loop-mediated isothermal amplification (LAMP). LAMP is rapid and uses a single temperature; thereby the reaction requires only a heat block or water bath, and thus can be performed in local settings. The current research also included inner loop primers to the LAMP to fasten the reaction time from 30 to 10 minutes.**

Keyword **- *Chlamydia trachomatis*, loop-mediated isothermal amplification, sexually transmitted disease**

I. Introduction

Chlamydia trachomatis represents the most common bacterial cause of sexually transmitted disease (STDs) worldwide. *C. trachomatis* is Gram-negative obligate intracellular human pathogen, and can be categorized into two major pathobiotypes: ocular biovar is associated with infectious blindness, and urogenital biovar associated with STDs. Mild acute symptoms 1–3 weeks following infection include dysuria, abdominal pain and abnormal vaginal discharge [1]. Additionally, *C. trachomatis* infections are often asymptomatic and long term, and the chronic infection is associated with serious disease outcomes, such as pelvic inflammation disease, ectopic pregnancy, and infertility in females and males, early diagnosis is essential for the timely treatment of *C. trachomatis*-infected women to prevent the development of sequelae and the transmission of *C. trachomatis* infection to susceptible individuals.

Polymerase chain reaction (PCR) has been established as the standard method for the *C. trachomatis* detection [2]. However, PCR is high cost, and need special equipments, thermocycling machines and electrophoresis[3,4]. This method alse requires handling of ethidiumbromide, which is carcinogenic.

Loop-mediated isothermal amplification (LAMP) is a recent technique for targeted nucleotide amplification at high sensitivity and specificity comparable to PCR, the LAMP reaction could be done in a very short amount of time and one temperature is required [5]. The only necessary equipment is a heating block or hot water bath. Unlike PCR, LAMP allows the amplification of specific DNA sequences in less than 1 hr, and the results can be observed by naked eyes through the clumping of the product as white precipitates [6]. With high specificity, high sensitivity, rapidity and simplicity, LAMP has been applied for many on-site detection of bacteria, virus, fungi and other parasitic diseases in humans [7].

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II. Materials and methods

(1) Sample collection and DNA extraction

Cervical swab samples were collected during 2011-2012 by clinicians, and transported in M4RT medium (Illinois, USA) [8]. After that, the samples were DNA extracted according to High Pure Template Preparation Kit instruction (Roche Diagnostics Corporation, Indianapolis, IN), and measured DNA quality and DNA concentration by A260/A280 and A260 spectrophotometer.

TABLE 1: Primers for PCR and LAMP detection of *C. trachomatis*

Primer name	**Primer sequence (5′ -> 3′)**
Ct.ompA.MVF3	TGTAAAACGACGGCCAGTGCCCGTGCAGCTTTGTGGGAATGT
Ct.ompA.220DR	GCGCTCAAGTAGACCGATATAGTA
CTompA_LAMP_FIP	TTAACTCCAATGTAAGGAGTGAACA-ATGCCTCTATTGACTACCAT
CTompA_LAMP_BIP	GGTCT(A/C)GAG(T/C)AAGTTTTGATGCCG-CAAGAT(T/A)GCTT(C/T)AGCCAATT
CTompA_LAMP_F3	GAACAGA(A/T)GC(T/A)GCGACAG
CTompA_LAMP_B3	(C/T)GGGTTTAGAGTAGT(G/A)A(C/T)ATC
CTinnerloop_LF	GGCTAAACTTGCTTGCCACT
CTinnerloop_LB	ATCCGTATCGCTCAGCCTA

(2) Detection of *C. trachomatis* by PCR and agarose gel electrophoresis

The standard method for PCR of the gene encoded for a major outer membrane protein (*ompA*) was performed. The *C. trachomatis ompA* PCR primers were Ct.ompA.MVF3 and Ct.ompA.220DR in Table 1 [9]**.** PCR was performed in 25 µl reaction mixture containing 100 ng of template DNA, 0.3 µM of forward and reverse primers, and 12.5 µl TakaRa PCR Amplification kit. The thermocycling profile was 94°C of 3 min; 35 cycles of 94°C 0.45 min, 45°C 1 min and 72°C 2 min, followed by a final extension at 72°C 10 min. To visualize the product, gel electrophoresis was required. For *C. trachomatis* serovar identification, sequencing of the *ompA* at Macrogen Inc. (Seoul, Korea) was performed.

(3) Design for LAMP primers

LAMP primers were designed based on all serovars of *C. trachomatis ompA*, using Primer Explorer V4 program (http://primerexplorer.jp/elamp4.0.0/index.html). The forward inner primer (FIP), backward inner primer (BIP), two outer primers (F3 and B3), and inner-loop forward primer (LF) and inner-loop backward primer (LB) were described in Table 1. Specificity of the primers was confirmed by blastn analyses.

(4) LAMP assay

The reaction was performed in 25 µL of reaction mixture containing 100 ng of DNA, 0.2 µM each of the F3 and B3 primers, 1.6 µM each of the FIP and BIP primers, 1.4 mM of dNTP mix (SibEnzyme Ltd., Novosibirsk, Rassia), 0.3 M betaine (Sigma–Aldrich, St. Louis, MO, USA), 6 mM MgSO4, 8 U of Bst DNA polymerase (large fragment; New England Biolabs Inc., Beverly, MA, USA) along with 1X of thermoPol^{TM} Reaction Buffer (New England Biolabs Inc., Beverly, MA, USA). The LAMP reaction mixture was incubated at 59, 61, 63 or 65°C temperatures, and over a range of times (30, 35, 40, 45, 50 and 60 min) to optimize the reaction conditions for positive reactions. The product was heated at 80°C for 4 min to terminate the reaction. LAMP products were observed by naked eyes, and also subjected to 2% agarose gel electrophoresis.

The mixture of LAMP reaction with inner loop primers was as previously stated, with 1.6 µM each of the LF and LB primers. The LAMP with inner loops reaction mixture was incubated at temperatures (59, 61, 63 and 65°C) and over a range of times (4, 6, 8, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, 30, 32.5 and 35 min) to optimize the reaction conditions for positive reactions.

(5) Determination of LAMP specificity

C. trachomatis serovars D, E, F, G, H and K DNA was used as positive controls, while the DNA from human papillomaviruses (HPV), *Neisseria gonorrhoeae*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* were used as negative controls. Reaction

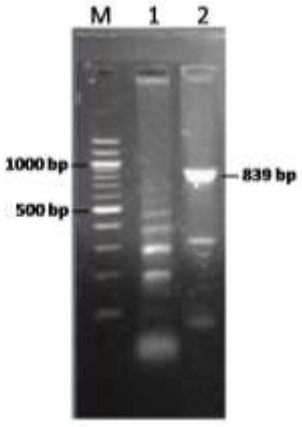

Fig. 1. Agarose gel electrophoresis showing positive *C. trachomatis ompA* LAMP (lane 1) and PCR (lane 2) results. M represents 100 bp DNA Ladder (New England Biolabs Inc., Beverly, MA, USA)

mixture containing distilled water was used as the other negative control. After LAMP, the amplicons were analyzed by naked eyes and 2% agarose gel electrophoresis.

III. Results

(1) Effectivity tests of PCR using established protocol [9] and LAMP using our designed primers

Established PCR primers and our designed LAMP primers based on the *C. trachomatis ompA* were successfully amplified *C. trachomatis* serovars D, E, F, G, H and K, and not in negative controls (HPV, *N.gonorrhoeae*, *S. epidermidis* and *S. saprophyticus*). All reactions contained 100 ng DNA template. Electrophoretic gel showed positive PCR product at 839 bp and LAMP product as smear of diversity bands (Fig.1).

(2) Optimization of LAMP conditions

Different The LAMP conditions were tested, including incubation temperatures of 59-65°C and incubation period of 30-60 minutes (Fig. 2). All four temperatures tested gave positive results, but the clearest and strongest bands were obtained at 61 °C, following by also tested, and 59°C, 65°C and 63°C, respectively. Different incubation periods were 30 min (61 °C) was found the minimum time to detect *C. trachomatis* by LAMP. Note shorter incubation periods (20 and 25 min) were tried, and yielded no result (data not shown).

(3) Optimization of conditions for added inner-loop primers

Inner loop primers, were designed and included in LAMP to speed up the LAMP reaction [16]. The amplification time now could be shorten to 10 min in

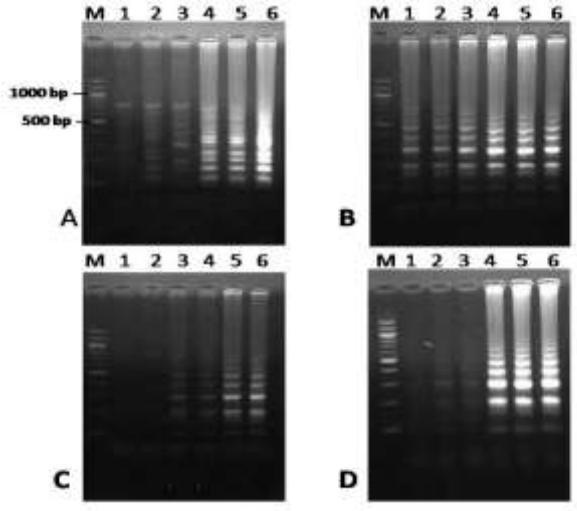

Fig. 2. LAMP without inner loop primer at different incubation temperature and time. A, the reaction temperature at 59°C; B, 61°C; C, 63°C; and D, 65°C. M represent 100 bp DNA Ladder (New England Biolabs Inc., Beverly, MA, USA). Lane 1-6 represent incubation time at 30, 35, 40, 45, 50 and 60 minutes, respectively.

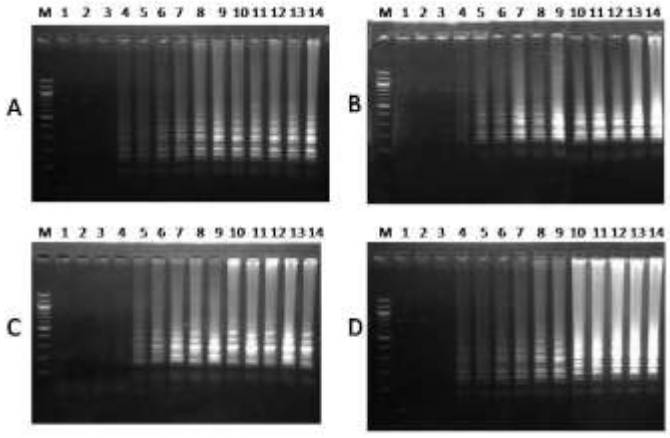

Fig. 3. LAMP with inner loop primers at different incubation temperature and time. A, the reaction temperature at 59°C; B, 61°C; C, 63°C and D, 65°C. M represent 100 bp DNA Ladder (New England Biolabs Inc., Beverly, MA, USA). Lanes 1-14 for incubation time at 4, 6, 8, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, 30, 32.5 and 35 minutes, respectively.

incubation temperature at 59, 61 and 65°C, respectively. The amplification at 63°C was shown at 12.5 min (Fig. 3). Fig. 4 showed the LAMP with inner loop primers reaction that could be observed as white precipitate by naked eyes.

(4) Determination for the specificity of the LAMP without and with inner loop primers

LAMP without inner loop primers were positive for all tested *C. trachomatis* serovars (D, E, F, G, H and K), and negative for all other microbes tested: HPV, *N.gonorrhoeae*, *S. epidermidis* and *S. saprophyticus* (Fig. 5). However, the LAMP with inner loop primers gave the positive results only in serovars D, G, H and K (Fig.6).

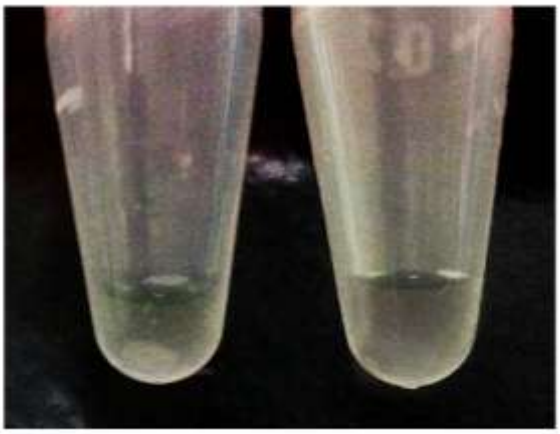

Fig. 4. LAMP with inner loop primers reaction observed by nake eyes (left). Right represent the reaction with *S. epidermidis* (negative control).

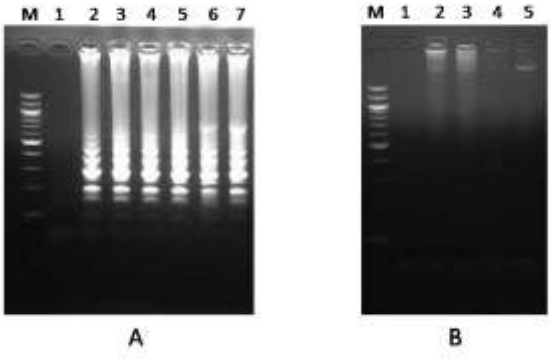

Fig. 5. Specificity tests for LAMP without inner loop primers at 61°C and 60 min incubation. A, *C. trachomatis* serovars D, E, F, G, H, K (lane 2-7), respectively. B, negative controls, including lane 2, HPV; lane 3, *N.gonorrhoeae*; lane 4, *S. epidermidis* and lane 5, *S. saprophyticus*. Lane M represents 100 bp DNA Ladder (New England Biolabs Inc., Beverly, MA, USA). Lane 1 represents no template control.

IV. Discussion

This research showed that our designed LAMP without and with inner loop primers could successfully detect only *C. trachomatis* (Figs. 5 and 6). The design of the inner primers allowed hybridizing to the target DNA sequence, and producing a dumbbell-shaped DNA. Then this special structure uses itself as a template for DNA synthesis to further synthesize stem-loop DNA, to fasten the reaction time [10-17]. Optimization assay showed 61°C the appropriate incubation temperature for this *C. trachomatis ompA* LAMP (Figs. 2 and 3).

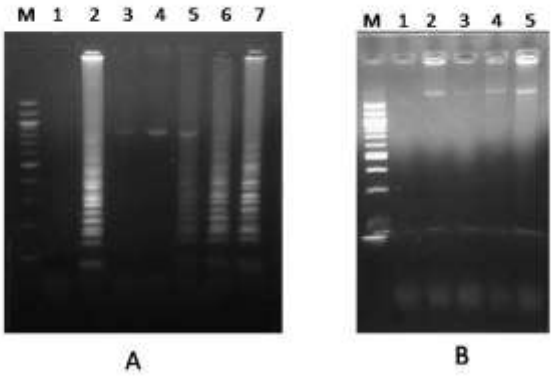

Fig. 6. Specificity tests for LAMP with inner loop primers at 61°C and 10 min incubation. A, *C. trachomatis* serovars D, E, F, G, H, K (lane 2-7), respectively. B, negative controls, including lane 2, HPV; lane 3, *N.gonorrhoeae*; lane 4, *S. epidermidis* and lane 5, *S. saprophyticus*. Lane M represents 100 bp DNA Ladder (New England Biolabs Inc., Beverly, MA, USA). Lane 1 represents no template control.

The LAMP, without and with inner loop primers, also showed the faster method than PCR, which requires method 3.5 hrs (fig. 1). In addition to the simplicity of the LAMP reaction without the need for expensive thermocycling machine, the ability to observe results by naked eyes via the presence of white precipitates (Fig. 4) confirmed another advantage of LAMP for on-site diagnostic test [18-21]. Moreover, the LAMP proved relatively high specificity, as no cross-reaction with other pathogens. Indeed, all the designed LAMP primers except the inner loop primers were checked against the GenBank database, and found the primers should be only specific for *C. trachomatis* serovars (data not shown). Subsequently, the lower specificity of the LAMP with added inner loop primers might be improved by selection of the new inner loop primers that were also conserved for *C. trachomatis* serovars D, G, H and K. Together, LAMP method was specific for *C. trachomatis*, inexpensive, short assay time, and potential for local and point-of-care diagnostic test.

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