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DEVELOPMENT OF REAL-TIME LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR DETECTION OF COXIELLA BURNETII

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ABSTRACT

Background: Real-time loop mediated isothermal amplification (LAMP) is a novel method for molecular detection that amplifies DNA with high specificity, efficiency, and rapidity under isothermal condition with portable fluorescent reader Genie II (Optigen, UK). *Coxiella burnetii* is a gram negative bacterium that causes zoonotic disease Q fever in human and ruminants worldwide. So, rapid detection is highly needful for counteraction of *C. burnetii* that can be detected by LAMP with high rapidity within 30-37 min, generally even within 30 min at 65-67°C and cycling reaction continues with accumulation of $2x10^{10}$ copies of target sequence.

Method: Positive control DNA of *C. burnetii* was amplified in conventional PCR with primers of insertion element IS1111a gene of *C. burnetii* and serially diluted in 10-fold series, performed in real-time LAMP. 244bp of IS1111a gene was inserted into plasmid vector with 10-fold serial dilution and performed by LAMP for detection of Q fever.

Results: To assess the development of LAMP, the recombinant plasmids were highly sensitive in LAMP and showed lower detection limit of gene as 0.02 copies per reaction. The concentrations were used as $2x10^{10}$ to $2x10^{-2}$ copies/µl.

Conclusion: The developed real-time LAMP assays targeting IS1111a markers of *C. burnetii* were shown to be highly specific, sensitive with efficient reproducibility. Copy numbers of *C. burnetii* isolate were reliably quantified. The result of present study indicates that the developed assay is able to detect pathogenic microorganism from small amount of template DNA.

INTRODUCTION

Coxiella burnetii is an obligate intracellular bacterium, a causative agent of Query fever (Q fever) inhumans and animals (Masala et al., 2004) such as cattle, goats, sheep, dogs, cats etc. *C. burnetii* is the most important reservoir in small wild rodents, lagomorphs, marsupials, monkeys, bats, birds and even reptiles and fish (Gardon et al., 2001; Sawyer et al., 1987; Serzov et al., 1999). Its infection has also been observed in ticks which are the most important vectors for playing an important role to transmit Q fever through bites or exposure. Most commonly Q fever is transmitted by inhalation of contaminated aerosol, contact with contaminated milk, meat, wool and particularly birthing products. Q fever is well recognized cause of abortions in ruminants and in pets. Infected animals can transmit the disease by when infected animals give birth, the environment contaminates by their infected amniotic fluid and placenta, and the organism can survive and present in soil and dust, for a long period in the environmental surfaces up to 60 days and can spread the disease long distances by air. This pathogen is extremely sustainable and virulent, and highly resistant to environment. Q fever has been described as two stages: an acute stage that presents with headaches, chills and respiratory symptoms, and an insidious chronic stage with inflammation of the inner lining of the heart named endocarditis (Karakousis et al., 2006). Treatment of the acute Q fever with antibiotics is immensely effective and the chronic stage is highly difficult to treat and can take up to four years to cure and resulting in high mortality (Walker, 1996). Apparently half of the infected individuals exhibit no symptoms (Anderson et al., 2011). So, Q fever has become the most important widespread zoonosis in the world. Rapid and sensitive diagnosis should be applied for the zoonotic disease in early phase otherwise the infection by the microorganism will occur acute Q fever that will turn into chronic Q fever, and death may occur. Conventional serodiagnosis requires sera from both acute and chronic infection, which are useless for swift diagnosis. In order to improve diagnosis for Q fever, real-time loop-mediated isothermal amplification (LAMP) assay is highly sensitive diagnostic method that has several advantages, including rapidity, high sensitivity, specificity, ease of application and low cost with effective outcome in less than under isothermal condition of 60-65°C. Several kinds of nucleic acid amplification

tools were developed including PCR based amplification (Saiki et al., 1985; Saiki et al., 1988), self-sustained sequence replication (3SR) (Guatelli et al., 1990), nucleic acid sequence based amplification (NASBA) (Compton, 1991), and strand displacement amplification, but LAMP is the most important diagnostic tool for the genetic disorder, infectious disease and genetic traits than others because it can amplify the DNA within 30 min. LAMP assay has been developed by *Notomi et al* (2000), with high sensitivity, rapidity, efficiency and specificity under isothermal condition, and it can be performed in a simple heating block. This method applies a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct regions on the target DNA following strand displacement DNA synthesis. Two inner and two outer primers and a loop primers has been used for detection of the insertion sequence transposes IS1111a gene of *C. burnetii* including a novel method LAMP.

MATERIALS AND METHODS

Amplirun® COXIELLA BURNETII DNA control (Vircell, Spain) [lyophilized DNA of *C. burnetii*, (Nine Mile Q strain (ATCC VR- 616)), ($1x10^4$–$2x10^4$ copies/µl once reconstituted)] was used as a sample.

Primer design for PCR

A conventional PCR was performed with Amplirun® COXIELLA BURNETII DNA control. The transposes gene of *C. burnetii* IS1111a insertion sequence that is present in 20 copies in the genome of the *C. burnetii* Nine Mile RSA493 strain (Gen Bank accession- AE016828.2) (Pan et al., 2013). An amplicon size of 244 bp of transposes gene IS1111a was constituted for identification. The forward and reverse primers for Q fever were defined as Cox_F and Cox_R respectively, are shown in Table 1. PCR amplification, that was performed in a 20 µl reaction system, using AccuPower® PCR PreMix (BIONEER, South Korea), 2 µl primers each (Cox_F and Cox_R) and 1µl of positive control DNA used as template. The PCR program was constituted as follows, initial denaturation at 95°C for 1 min, 30 cycles consisted of denaturation at 95°C for 15s, annealing at 58°C for 14s and elongation at 72°C for 30s. The 244 bp amplified PCR products were examined by electrophoresis on 1.5% agarose gels stained

with Redsafe™ Nucleic Acid staining solution. The PCR product was purified using the GENE ALL Expin™ gel and the identity of the amplified products was confirmed by DNA sequencing.

Primer design for real-time LAMP

Two sets of universal primers were designed using Primer Explorer V4 software [http://primerexplorer. jp, Eiken chemical Co., Ltd., Tokyo, Japan] against the repetitive sequence IS1111a of *Coxiella burnetii* RSA 493 (AE016828.2) based on conserved sequences. Sequences and positions of the repetitive sequence IS1111a, are shown in Figure 1 and Figure 2. Designed LAMP primer set 1 and LAMP primer set 2 that were defined as CB_01 and CB_02 respectively, are shown in Table 2 and Table 3, respectively. It was designed 5 primers (F3, B3, FIP, BIP and LF) for each set and that were forward outer primer (F3), backward outer primer (B3), forward inner primer (FIP) contains the complementary sequence of the F1 region and the sense sequence of the F2 region (F1c + F2) and backward inner primer (BIP) conducts the complementary sequence of the B1 region and the sense sequence of the B2 region (B1c + B2), forward loop primer (LF), applied those for the detection of IS1111a gene in LAMP assay. For developing LAMP assay, both 2 sets of primer for IS1111a gene and ran the reaction mixtures together on fluorometer Genie II. Sequencing analysis using BLAST (data not shown) .

TA cloning and preparation of standard plasmid

After PCR amplification of the transposes IS1111a gene of *C. burnetii* using Cox_F and Cox_R primers, TA cloning of the product (244bp) was carried out. The sequence for the LAMP between the F3 and B3 primer binding sites were expanded using primers Cox_F and Cox_R. For this purpose, the amplified PCR product was purified using the GENE ALL® Expin™ gel and the product including 6x loading dye (Fermentas) was re-electrophorsed on 1.5% agrose gel in TBE buffer, stained with Redsafe™ Nucleic Acid staining solution and visually observed by UV illumination. The identity of the amplified product was confirmed by DNA sequencing analysis using BLAST (data not shown). The purified PCR product IS1111a gene fragment with the length of 244 bp was ligated into pGEM®-T Easy vector (3015bp) by 1U of T4 DNA ligase according to work instructions which was obtained from Promega (Madision, WI USA) PCR cloning kit. Competent cells of *E. coli* JM109 were transformed with the ligation reaction

product. The transformed cells were incubated at 37°C for 24 h on Luria-Bertani (Merck, Darmstadt, Germany) medium containing 100 µl 100mM IPTG (isopropyl-beta-D-thiogalactopyranoside) (Sigma, St. Louis, MO, USA), 20µl 50mg/ml X-gal (5-bromo-4-chloro-3-indolyl beta Dgalactoside) (Sigma), and 15 µl ampicillin (Merck). Recombinant clones on the medium were identified by blue/white screening and white color colonies containing recombinant plasmid vector were selected for further experiment. After cloning, plasmids of the selected clones were maintained in *E. coli* cells for obtaining multicopy of plasmid. 20 µl of plasmid infected *E. coli* cells were inoculated into 20ml TSB (Trypic soy broth) media and incubated at 37°C for 24h, after that this step was repeated for subculture followed by previous terms. The subcultured plasmids were used as a stock solution. After centrifugation at 4000rpm, 1000µl TSB including glycerol were added in 200 µl stock solution for establishing plasmid glycerol stock for long term storage of plasmid and stored at -80°C for further purposes. 20µl of glycerol stock solution was inoculated into 45 ml of TSB media and incubated at 37°C for 18h with shaking. Then, the plasmids were purified by Macrogen Inc. (Seoul, south Korea) Midiprep kit according to manufactures protocol and 244 bp products and insertion sequence IS1111a gene containing recombinant plasmids were confirmed by PCR with primers (Cox_1 and Cox_2) (Figure 3A and B). After confirmation of the recombinant plasmid DNA was used as positive control, plasmid DNA in the LAMP assays and PCR amplification for determining the comparison of sensitivity between of these two assays with IS1111a plasmid DNA. Quantitative evaluation was generated based on this plasmid DNA.

Optimization of the LAMP reaction

The LAMP assay was performed in 25 µl of the final reaction volume. The reaction mixture contained 15 µl of 1x ISO-001 isothermal Master Mix (Optigen, United Kingdom) including G*sp*SSD large fragment DNA polymerase isolated from Geobacillas species, thermostable organic pyrophosphatase, optimized reaction buffer, $MgCl_2$, deoxynucleoside triphosphatase and a double stranded DNA binding dye (Optigen), 1 µl each of two outer primers F3 and B3, two forward and backward inner primers FIP and BIP, respectively and one loop primer LF, and 3 µl of *Coxiella burnetii* DNA control (Amplirun®) as a template, and 2 µl of deionized sterile distiled water (BIONEER). In the case of plasmid purified DNA

that was serially diluted and used 1 µl of the dilutions for each reaction including reaction mixture as above with 4 µl of deionized sterile distal water (BIONEER). All the LAMP reaction mixtures were run in a real-time fluorometer Genie II (Optigen, UK) (Figure 4A, B and C) that is easy to use and determines the optimal temperature with the shortest amplification time including highest speed fluorescence reading and followed at the temperatures of 65°C to 67°C. All the LAMP assays were run for the time between 30 min and 37 min for *C. burnetii* DNA and plasmid purified DNA, respectively followed by heating and cooling steps of 98°C to 80°C at 0.05°C/s to allow reannealing of amplified DNA and Genie II displays annealing curve with annealing temperature values for each reaction. After amplification, the LAMP products were examined by electrophoresis on a 2% agarose gels in TBE (Trizma base, Boric acid, EDTA) buffer (BIONEER), stained with Redsafe™ Nucleic Acid staining solution and visual inspection with under UV illumination .

Standard curve analysis

To establish the minimum copy number (lower detection limit) of the target gene IS1111a insertion sequence detectable by real-time LAMP, positive control plasmid DNA was used. The standard curve for LAMP was constituted by using serial dilutions of plasmid DNA (at concentrations $2x10^{1^\circ}$ to 0.02 copies/µl) with deionized water. For each standard, the copy number was plotted against the threshold time. The resulting plot was analyzed by linear regression and there was a clear linearity between the threshold time and the initial amount of control DNA (Figure 5A and B) and positive control plasmid DNA (Figure 5C and D). The statistical significance of the r^2 values was analyzed by analysis of variance (ANOVA). Probabilities of less than 0.05 were considered statistically significant. A plot was generated with the threshold time versus the log of the initial template of control DNA and plasmid DNA copy number, and that showed a linear regression, with statistically significant regression coefficient: in the case of control DNA, $r^2 = 0.88$ ($P = 0.04$) , $r^2 = 0.99$ ($P = 0.01$) using primers CB_01 and CB_02 respectively, and obtained plasmid DNA copy number using primer set of CB_01 and CB_02 with regression values $r^2 = 0.83$ ($P = 0.0002$), $r^2 = 0.72$ ($P = 0.003$), respectively.

RESULTS

Sensitivity of the LAMP assay

The sensitivity of LAMP assay was evaluated by testing of ten-fold serial dilutions of C. *burnetii* DNA control and plasmid DNA containing IS1111a insertion element of *C. burnetii*, and the enzyme displays highest speed in a fluorescent LAMP reaction. The recombinant plasmid was quantified as 64.042 ng/µl of the concentration by a spectrophotometer. The sensitivity was assessed using serial ten folds dilution with deionized sterile distiled water (at concentrations of $2x10^{1°}$, $2x10^{9}$, $2x10^{8}$, $2x10^{7}$, $2x10^{6}$, $2x10^{5}$, $2x10^{4}$, $2x10^{3}$, $2x10^{2}$, $2x10^{1}$, 2, 0.2, 0.02 copy numbers per reaction, comparatively) of IS1111a plasmid DNA to evaluate the limit of detection and the reproducibility in LAMP and PCR assays. One µl of each concentration was used as a template for the LAMP and PCR reactions. The limit detection of LAMP (CB_01) and PCR were 10 copies/µl showed the similar results and the PCR assay (10^{4} to 10^{1} copies/µl) was 10-fold more sensitive including primer CB_02 (10^{4} to 10^{2} copies/µl) with control DNA (Figure 6A, B and C). In the cases of recombinant plasmids in LAMP and PCR assays, 0.02 and $2x10^{2}$ copies/ µl in each reaction, respectively (Figure 7A, B and C); therefore the real-time LAMP is 200-fold more sensitive than conventional PCR for the detection of *C. burnetii*. The copy numbers were calculated using the formula below:

$$\text{Copies/µl} = 6.02\times10^{23} \times (\text{ng/µl} \times 10^{-9})/ (\text{DNA length} \times 660)$$

To quantify the copy number of the plasmid standard curve was generated by plotting the Tt (Time threshold) values against log copy number and linear regression was calculated using Microsoft Excel program. Each detection assay was performed in triplicate.

Specificity of the LAMP assay

To establish the specificity of real-time LAMP, 2 sets of primers of transposes gene of *C. burnetii* IS1111a insertion sequence were used in PCR and LAMP. These primers amplified the control DNA and plasmid DNA in two types of diagnostic tools, and indicated the positive results in each reaction. Therefore, Q fever can be detected in LAMP and PCR, the positivity obtained using the primers in initial

amount of DNA and plasmid DNA used as a template. So this IS1111a sequence is highly specific in LAMP for the determination of Q fever.

Detection of IS1111a gene with CB_01 and CB_02 primers

The temperature and primer concentration for LAMP were optimized for the rapid detection of *C. burnetii* positive control DNA and positive control plasmid DNA. Gene with 198bp and 189bp fragment size was optimized with primer set CB_01 and CB_02, respectively with Genie II fluorometer simultaneously. The Genie II shows the amplification signals with fluorescence, and ending of the run and displays the time for positive results and temperature (*Tm*) for each reaction, for the detection of *C. burnetii* from control DNA with primer CB_01 amplified in amplification time that ranged from 24:15 to 29:15 min (mean time 26:18 min) annealing Tm that ranged from 83.01 to 83.33°C, (mean 83.18°C), in the case of CB_02 primer, the amplification time that ranged from 16:45 to 23:15 min, (mean 26:8 min) and Tm ranged 83.07 to 83.31°C (mean 83.19°C) that are shown in Table 4. Control DNA was serially diluted into a ten-fold series and the detection of limits were 10^4 to 10^1 copies/µl with CB-01 primer, and 10^4 to 10^2 copies/µl with CB_02 primer, and dilutions were evaluated including both primers and run on for 30 min at 65°C, and performed in PCR also with primer Cox_F and Cox_R, obtained 10^4 to 10^1 copies/µl for comparing the detection limit between LAMP and PCR.

To examine the sensitivity of LAMP assay with positive control plasmid DNA that was serially ten-fold diluted and run with fluorescence detector Genie II and it displays the amplification time that measured from 16:00 to 34:15 min (mean 28:26 min) and Tm values that ranged from 82.27 to 87.17°C (mean Tm 83.33°C) with CB_01 primer, and the amplification time that measured from 12:45 to 27:30 min (mean 23:8 min) and Tm values that ranged from 81.1 to 83.21°C (mean Tm 82.94°C) using CB_02 primer (Table 5). The overall mean time to positivity for all positives for both sets of primers (to concentrations $2x10^{1^\circ}$ to 0.02 copies/µl), and LAMP reaction was set to run for 35 min and 37 min at 67°C for using CB_01 and CB_02 primers, respectively.

Table 1. Primer sequences used for amplification of IS1111a gene in PCR

Name and type	Position	Amplicon size	Sequence (5'-3')
Cox_F	466706-466949	244 bp	ACACGCTTCCATCACCACG
Cox_R			TGAAATGGACCCACCCCTT

Table 2. A primer set CB_01 for amplification of IS1111a gene in LAMP. LAMP primers; OF, OR, IF, IR and LF indicate outer forward, outer reverse, inner forward, inner reverse and loop forward respectively.

Primer name	Type	Length(n)	Amplicon size	Sequence (5'-3'), position (466726-466922)
F3	OF	18	198 bp	AGCCCACCTTAAGACTGG
B3	OR	18		CGTCATAATGCGCCAACA
FIP(F1c-F2)	IF	45		GATGAGTGGGGTAAAGTGATCTAC-CTACGGTGGATACATACTGAG
BIP(B1c-B2)	IR	47		TTCCACACAGTTGAAAAACATCTTT-AAAAAAGGAGAAGGTCCATGAA
LF	LF	19		ACGAGACGGGTTAAGCGTG

Table 3. A primer set CB_02 for amplification of IS1111a gene in LAMP

Primer Name	Type	Length(nt)	Amplicon size	Sequence(5'-3'), position (466734-466922)
F3	OF	18	189 bp	TTAAGACTGGCTACGGTG
B3	OR	18		CGTCATAATGCGCCAACA
FIP(F1c-F2)	IF	42		TGTGTGGAATTGATGAGTGGGG-TACATACTGAGCACGCTTAA
BIP(B1c-B2)	IR	50		AACATCTTTTGCAATATCAACACCC-TATTTTCAAAAAAAGGAGAAGGTCC
LF	LF	21		AAGTGATCTACACGAGACGGG

Table 4. Sensitivity of positive control DNA containing IS1111a gene with amplification time and annealing temperature of LAMP in Genie II.

No. of positive control DNA (copies/µl)	**Amplification time (min:s), Tm(°C)**	
	Primer CB_01	**Primer CB_02**
10^4	24:30, 83.26	16:45, 83.19
10^3	24:15, 83.33	19:30, 83.31
10^2	27:15, 83.01	23:15, 83.07
10^1	29:15, 83.15	

Table 5. Sensitivity of positive control plasmid DNA containing IS1111a gene with amplification time and annealing temperature of LAMP in Genie II.

No. of positive control plasmid DNA(copies/µl)	Amplification time (min:s), Tm(°C)	
	Primer CB_01	Primer CB_02
$2x10^{1°}$	16:00, 83.12	12:45, 83.21
$2x10^{9}$	19:30,82.93	17:15, 83.18
$2x10^{8}$	22:45, 83.07	19:45, 83.11
$2x10^{7}$	25:30, 82.97	22:45, 83.06
$2x10^{6}$	26:00, 86.10	24:45, 83.10
$2x10^{5}$	31:15, 82.69	27:15, 83.07
$2x10^{4}$	31:15, 82.66	27:30, 83.05
$2x10^{3}$	31:15, 82.70	25:45, 83.19
$2x10^{2}$	33:45, 82.69	27:15, 83.04
$2x10^{1}$	34:15, 82.33	27:15, 83.05
$2x10^{˚}$	34:15, 82.27	27:15, 83.16
0.2	33:15, 82.66	26:30, 83.00
0.02	30:00, 87.17	26:15, 83.09

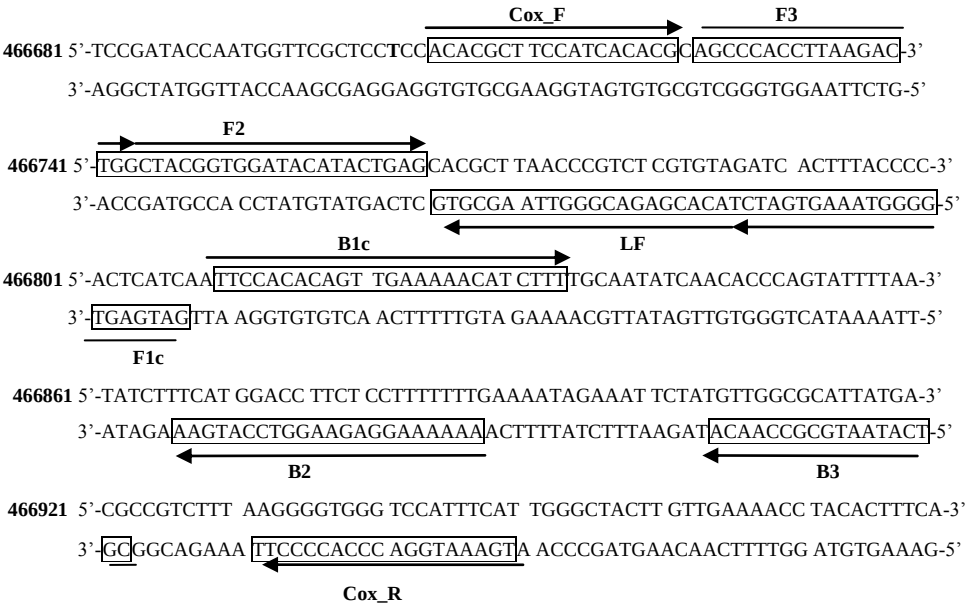
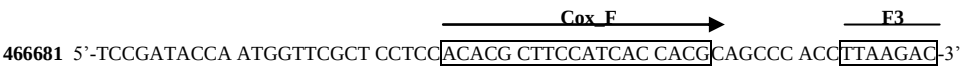


Figure 1. Location and sequence of PCR and LAMP (CB_01): Primer (CB_01) targets for IS1111a gene of *Coxiella burnetii*. Primer binding sites of Cox_F and Cox_R used in PCR. PCR primers and 5 LAMP primers: F3, B3, FIP (F1c-F2), BIP (B1c-B2) and LF, arrows indicate including box the direction of the pathway. GenBank accession number AE016828.2.



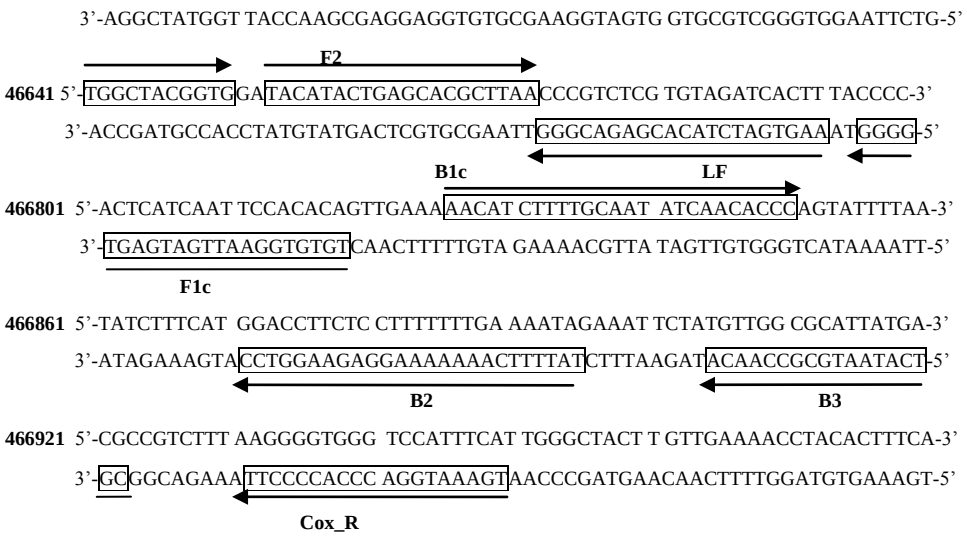


Figure 2. Location and sequences of PCR and LAMP (CB_02): Primer (CB_02) targets for IS1111a gene of *Coxiella burnetii*. Primer binding sites of Cox_F and Cox_R used in PCR.PCR primers and 5 LAMP primers: F3, B3, FIP (F1c-F2), BIP (B1c-B2) and LF, arrows indicate including box the direction of the pathway. GenBank accession number AE016828.2 .

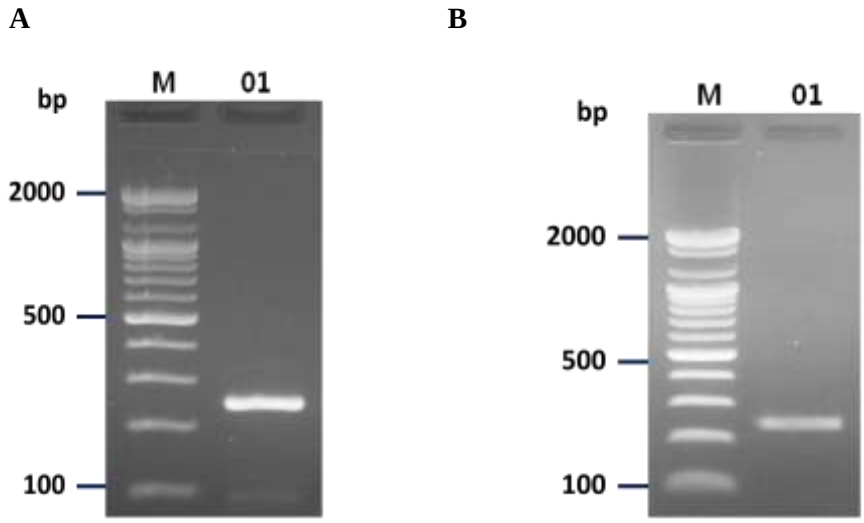
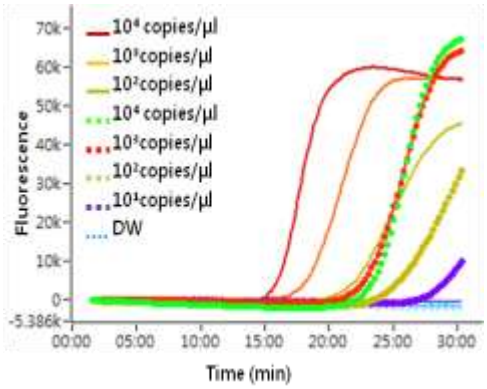


Figure 3. Identification of PCR amplified products (244bp): Primers Cox_F and Cox_R using agarose gel electrophoresis stained by Redsafe. M 100bp DNA molucular weight markers**.** A. Positive control DNA of transposes IS1111a gene of *Coxiella burnetii.* B. Transposes IS1111a gene Positive control plasmid DNA of *C. burnetii* .

A



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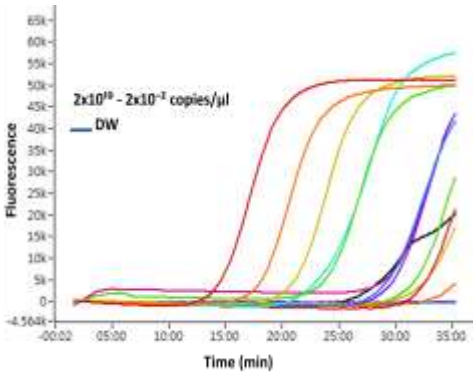

C

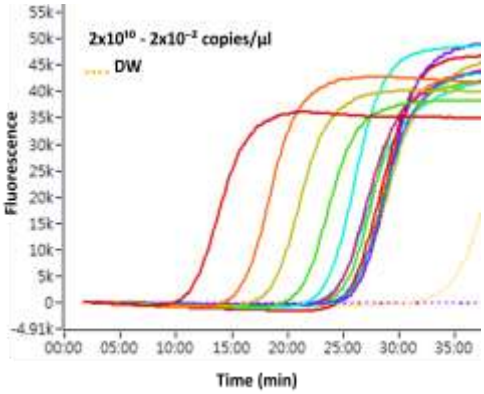

Figure 4. Sensitivity detection of DNA and plasmid DNA in fluorescence detector Genie II: Sensitivity detection of transposes IS1111a gene (A) and recombinant plasmids of transposes IS1111a gene with 10-fold serial dilution using CB_01 and CB_02 of LAMP primers in fluorescence detector Genie II (B) and (C) respectively.

A **B**

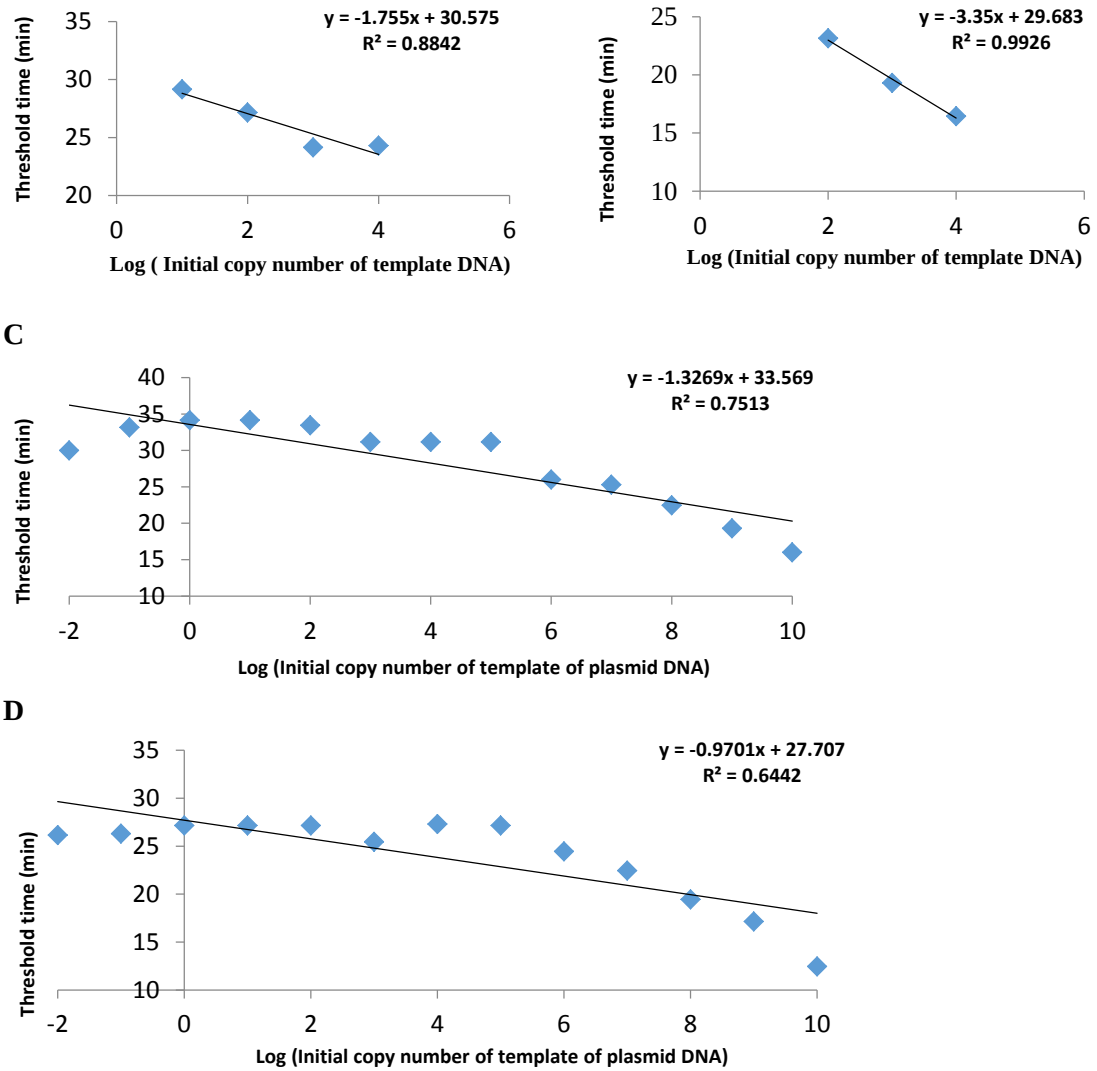

Figure 5. Standard curves of 10-fold serial dilutions of control DNA and plasmid DNA performed in real-time LAMP: Plots represent the mean threshold time against the log of the input DNA (A, B) and plasmid DNA (C, D) fit a linear regression showing R^2 value copy number of 10^4 to 10^1 copies/µl (**A**) and 10^4 to 10^2copies/µl (B) with primers CB_01 and CB_02 respectively**,** and copy number of $10^{1°}$ to 10^{-2} copies/µl (C) and (D) with primers CB_01 and CB_02 respectively.

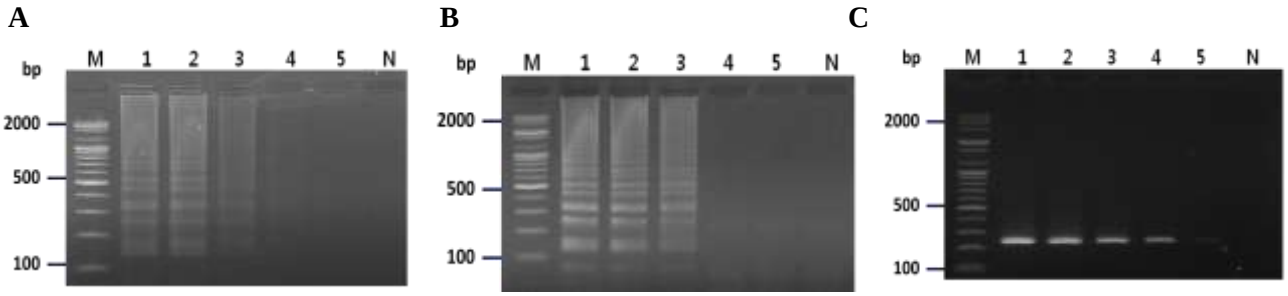

Figure 6. Analytical sensitivity of LAMP and PCR with control DNA: Sensitivity of LAMP (A and B) and PCR (C), was performed using 10-fold serial dilutions of positive control DNA containing IS1111a gene of *C. burnetii*, including primers CB_01 and CB_02 for the lower limit detection of *C. burnetii* (A) and (B) respectively. 244bp PCR products with primers Cox_F and Cox_R (C). Showing a comparison of sensitivity between LAMP and PCR.using agarose gel electrophoresis stained by Redsafe. Lanes 1-5 represent 10^4, 10^3, 10^2, 10^1, 10^0 copies/µl DNA. Lane M. 100bp DNA molucular weight markers and lane N, negative control.

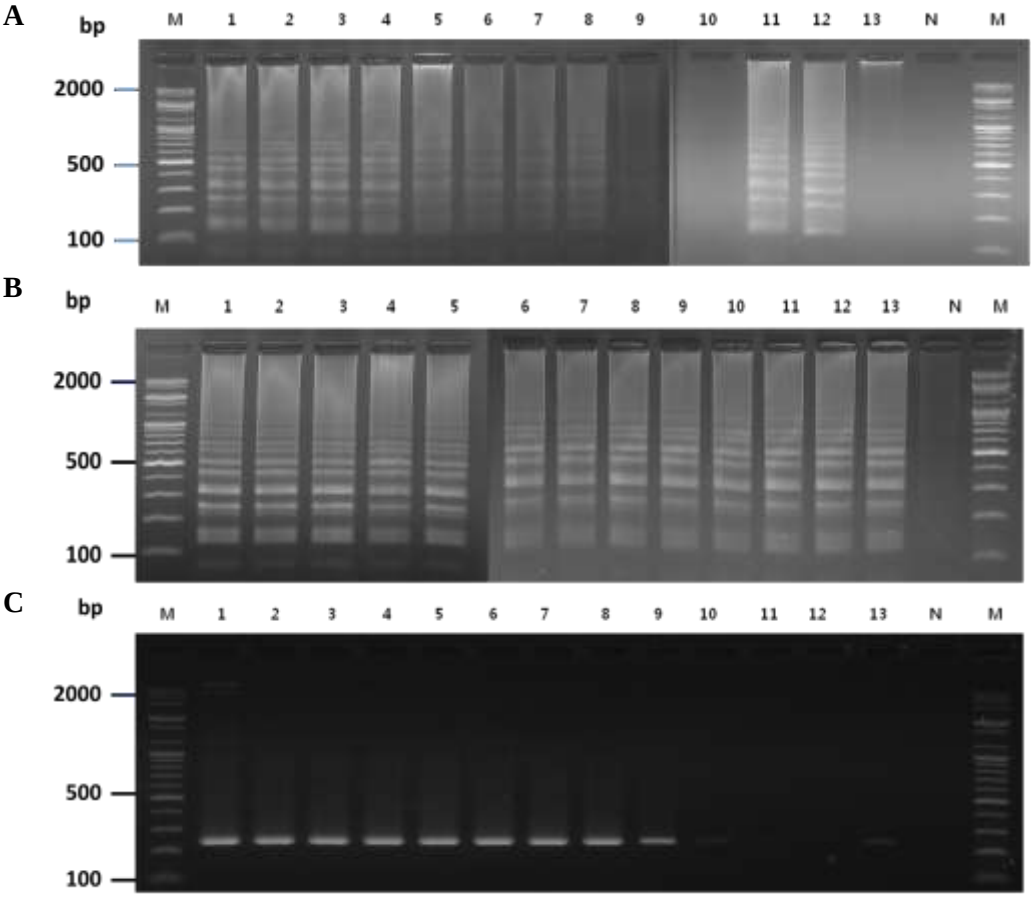

Figure 7. Analytical sensitivity of LAMP and PCR with plasmid DNA: Sensitivity of LAMP (A, B) and PCR. (C), was performed using 10-fold serial dilutions of positive control plasmid DNA containing IS1111a gene of *C. burnetii*, including primers CB_01 and CB_02 for the lower limit detection of *C. burnetii* (A) and (B) respectively. 244bp PCR products with primers Cox_F and Cox_R (C). Showing a comparison of sensitivity between LAMP and PCR using agarose gel electrophoresis stained by Redsafe. Lanes 1-13 represent $2x10^{1°}$, $2x10^9$, $2x10^8$, $2x10^7$, $2x10^6$, $2x10^5$, $2x10^4$, $2x10^3$, $2x10^2$, $2x10^1$, 2, 0.2, 0.02 copies/µl per reaction. Lane M 100bp DNA Ladder and lane N, negative control (DW).

DISCUSSION

Q fever is the most widespread zoonosis, and it is difficult to distinguish from other febrile disease because of lack of specific clinical manifestations, and commonly required by infectious aerosols or contaminated dust. So, it's accurate diagnosis is necessary with a congruous diagnostic tool within a shorter time. LAMP can be performed in fluorescence reader Genie II that can provide a sensitive result within 30 min, and display time threshold values against fluorescence under isothermal Tm at 65°C.

In this study, a LAMP assay has been developed for rapid detection of transposes IS1111a gene of *C. burnetii* with an analytical sensitivity and showed a positive results within 37 min. Optimization of the amplification temperature was 67°C and concentrations of the primers were for F3 and B3 primers at 0.2 μM, for FIP and BIP primers at 0.8 μM and for LF primer at 0.4 μM. A commercially available Isothermal mastermix (ISO-001) containing an improved G*sp*SSD large fragment DNA polymerase (Optigen, United Kingdom) and it decreased amplification times. The LAMP reaction was accelerated by the addition of two primers, termed loop primers LB and LF. For this study, one loop primer (LF) has been used.

The developed LAMP can amplify *C. burnetii* containing multicopy transposes IS1111a gene using two sets of primers simultaneously from positive control DNA. Resulting the copy numbers were 10^4 to 10^1 for primer CB_01 and 10^4 to 10^2 for primer CB_02. The lower limit of detection of copy number is 10 copies/μl when using primer CB_01 and the amplification time that ranged 24:30 to 29:15 min (mean time 26:18 min) and another LAMP reaction using CB_02 primer Genie II displays the amplification time ranging 16:45 to 23:15 (mean time 19:6 min) including copy number is 100 copies/μl for the detection of Q fever. In the observation of two primers coupled with LAMP reaction the amplification time (mean time 26:18 min) increased using CB_01 primer can detect the minimum copy number of target gene (10 copies/μl) and using CB_02 primer the amplification time (mean time 19:6 min) decreased with showing limit 100 copies/μl as a lower detection. Therefore, LAMP can perform the detection of Q fever within 30 min under isothermal condition using two sets of primers of the target gene simultaneously. The limit

detection when using primers CB_01 and CB_02 for the target gene were 10 and 100 copies per reaction, respectively. But CB_01 primer required longer amplification time then CB_02 although LAMP can be able to detect 10 copies per reaction whereas 100 copies per reaction using CB_02 primer with a shorter amplification time. So, pathogen can be detected by LAMP including a shorter amplification time.

Sensitivity testing for this study, LAMP detected 0.02 copies per reaction as a lower limit of detection from positive control plasmid DNA when that was serially diluted in ten-fold series including two sets of primers. Amplification time 16:00 to 34:15 min (mean 28:26 min) was longer using CB_01 primer and amplification time 12:45 to 27:3 min (mean 23:8 min) was shorter using CB_02 primer and LAMP amplified the plasmid DNA using both of two sets of primers detecting 0.02 copies/µl for each reaction that was scored as positive. So, LAMP is highly sensitive for rapid detection of the target gene.

The common transmission route of *C. burnetii* to humans and animals is considered to be through the inhalation of contaminated aerosols originated by animal products (Karagiannis et al., 2009; Madariaga et al., 2003; Parker et al., 2006; Schimmer et al., 2010), and generally for human infection, sheep and goats appear to be a more important risk factor than cattle and wild animals. The highest concentrations of *C. burnetii* in veterinary matrices are found in birth products, such as amniotic fluids and placentas (Arricau Bouvery et al., 2003; Masala et al., 2004), and in lower quantities in milk (Guatteo et al., 2007; Rodolakis et al., 2007) and blood (To et al., 1998). Therefore, the most likely route for *C. burnetii* to enter the environment is by shedding through placenta materials and amnion fluids, for instance, during the lambing season.

C. burnetii resides and multiplies in phagolysosomes and can survive long periods of time in infected cells without affecting viability (Raoult, 1990). Due to its persistence in the environment (Hackstadt, 1990; Janse et al., 2010) and its low infectious dose (Beneson et al., 1956), it has been classified as a category B bioterrorism agent by the centers for Disease control and Prevention (CDC) in Atlanta, United states (Madariaga et al., 2003).

The diagnosis of Q fever, both in humans and animals, is based mainly on serology. Serological methods used for the detection of *C. burnetii* are indirect immunofluoroscence, complement fixation, or Enzyme

linked Immunosorbent assay (Rousset et al., 2007; Slabå et al., 2005). A drawbacks of these techniques is that diagnosis is delayed because *C. burnetii*-specific antibodies appear 2-3 weeks after infection and can be detected months after an infection. Serology is, for these reasons, less suitable for direct transmission and source-funding studies for *C. burnetii* infection (Bruin et al., 2011); for this purpose real-time LAMP is more suitable than others. For the purpose of diagnosis, conventional PCR and real-time PCR are the effective tools but these require more time to amplify the target gene. LAMP is a nucleic acid amplification method that depends on autocycling strand-displacement DNA synthesis with G*sp*SSD LF DNA polymerase enzyme. The amplification products are stem-loop DNA structures with several inverted repeats of target and structures with multiple loops. LAMP is a novel method that can be applied in various kinds of microorganism detection and has been applied for viral (Bista et al., 2007; Parida et al., 2004), bacterial (Enosawa et al., 2003; Iwamoto et al., 2003), fungal (Inåcio et al., 2008), parasitic pathogens (Bakheit et al., 2008; Kuboki et al., 2003) and Rickettsial pathogens (Nakao et al., 2010) detection. It requires only one isothermal mastermix that contains DNA polymerase and/or *Bst* DNA polymerase enzyme and four primers that recognize six distinct target regions. This tool originates a large amount of amplified product, resulting in convenient detection, like detection by visual inspection of the turbidity that can be seen by the naked eye or fluorescence of the reaction mixture (Mori et al., 2001), and the reaction can be constituted under isothermal conditions, as well as there is no time consuming drawbacks in LAMP with the quality of high sensitivity, rapidity, specificity.

CONCLUSION

The developed real-time LAMP assays targeting IS1111a markers of *C. burnetii*, and more convenient for detection of pathogen with ease of use. The assays were shown to be highly specific, sensitive with efficiently reproducible. Copy numbers of *C. burnetii* isolate were reliably quantified. The result of this present study indicates that it is strongly able to detect pathogen from small amount of template DNA.

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