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A Real-time Quantitative Loop Mediated Isothermal Amplification Assay Targeting *com1* Gene of *Coxiella burnetii*

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Abstract

Background & Objectives: The present study designed a loop mediated isothermal amplification (LAMP) method to rapidly detect *Coxiella burnetii* (*C. burnetii*) and develop a sensitive Real-time quantitative LAMP (Q-LAMP) assay to quantify Q fever.
Materials & Methods: Primers were specifically designed for use in targeting the *com1* conserved gene of *C. burnetii* to carry out LAMP detection of the agent causing Q fever. After obtaining the LAMP reaction, the sensitivity of the method was assessed by preparing a serial ten-fold dilution of a plasmid carrying *com1* gene.
Results: The assay's sensitivity for the visual detection of changes in turbidity or turbidimeter and electrophoresis of agarose gel were 0.4 fg and 0.04 fg, respectively. Hence, the lower limit of detection was 120 and 12 copies of the gene detected in 60 min. The results of this study were indicative of the simplicity, rapidness, sensitivity, and specificity of the LAMP assay for *C. burnetii* detection and a probable improvement of the diagnostic procedure used in clinical laboratories.
Conclusion: The assay specificity was assessed using *Coxiella* genomic DNA and a panel of Gram-negative and Gram-positive bacteria. The LAMP assay was shown to be highly specific for detection of *Coxiella* without any observable amplification products from non-*Coxiella* organisms.

Keywords: *Coxiella burnetii*, Q fever, Loop mediated isothermal amplification (LAMP)

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Introduction

Coxiella burnetii (*C. burnetii*), the causative agent of Q fever in human and coxiellosis in animals, is an obligate intracellular bacterium belonging to the family Coxiellaceae (1-3). This bacterium has been relatively recently relocated to the order Legionellae's, class Gammaproteobacteria (4). The microorganism is transmitted to humans through the respiratory and gastrointestinal tracts and the skin. Inhalation of contaminated dusts or droplets carrying *C.burnetii* shed by infected animals is the main route of human infection (5, 6). Q fever is an acute and chronic febrile disease in humans. Although the

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disease is a self-limited febrile illness, but severe clinical complications such as endocarditis, lung issues, liver damage and meningitis have been reported (7, 8). Recently *C.burnetii* has been detected in periprosthetic tissue and synovial fluid by PCR and culture so Q fever may be tracked in patients with prosthetic joint infection with no detected pathogen (9). As a zoonotic pathogen, *C.burnetii* also causes abortion and stillbirth in ruminants, dogs, and cats (10). It causes substantial economic loss when infecting livestock (10-12). Simplicity of droplet distribution, ecological sustainability, and low ID50 make *C.burnetii* a serious threat for militants and noncombatants (13, 14). *C. burnetii* is considered a category B potential bioterrorism agent (15). This bacterium has previously been used and mass-produced as a bioweapon under several biological warfare programs (16-17).

Although there has been little research conducted on human Q fever in Iran and other Middle East regions, a survey has shown a high prevalence rate of *C.burnetii* exposure in samples obtained from febrile patients in Iran (18). However various studies have shown *C.burnetii* in milk of cows and goats in several provinces (19-21).

Microbiological, molecular, and serological tests are current diagnostic methods for detecting Q fever (22). Molecular techniques such as PCR are useful for rapid detection of the bacterium in biological samples. Although pathogen isolation by culture is the definitive method of diagnosis, it is time-consuming, is not always reliable, and involves low sensitivity. Molecular methods such as PCR and real-time PCR are sensitive and specific methods to detect the bacterium but require costly equipment and skillful technicians (23, 24).

The LAMP assay is a gene amplification method characterized by the application of four to six primers that specifically recognize distinct regions on the target DNA. A LAMP reaction can be performed at a continuous temperature (60-65˚C) in less than an hour and requires no special equipment. It requires simple conditions for molecular detection of various microorganisms, including fungi, bacteria, parasites and viruses (23). In the present study we designed and developed a sensitive, specific and quantitative LAMP assay based on highly conserved region of single copy gene of *com1* for rapid detection of *C. burnetii*. This is to our knowledge the first study that describes a Q-LAMP assay to quantitatively detect *C. burnetii*.

Materials and Methods

Sequence alignments and primer design

Forty registered sequences of 27 KDa outer membrane protein (*com1*) gene of *C. burnetii* were initially obtained from the Gene Bank sequence database and a multiple sequences alignment was performed using CLC sequence viewer 6.4 software (CLC bio, Aarhus, Denmark) (Accession numbers not shown). A set of six *C. burnetti* -specific LAMP primers containing loop primers were designed based on the consensus sequence of the gene by online software program (Primer Explorer V4 software) from Eiken chemical (http://primerexplorer.jp/e/). *In silico* analysis using BLAST and Primer-BLAST on the NCBI server (http://www.ncbi.nlm.nih.gov/) confirmed the theoretical specificity of the designed primers. The primers were custom synthesized by Bioneer (Daejeon, Korea) and are listed in Table 1.

Table 1. Primer sequences used for *com1* gene of *Coxiella* in LAMP assay

Primers	Sequences (5'-3')
FIP-Cb-com1	5'-TGAAGACAACGCGGAGGTTTCCATGAATTCTGTTATTCAAGC-3'
BIP-Cb-com1	5'-AGAACTGCCATTTTTGGCGCATAATATTTTCCTTGTTTAGCGG-3'
B3-Cb-com1	5'-TTATTGGGCGTCGACACT-3'
F3-Cb-com1	5'-AATGTGGCCATTGCAAAG-3'
Loop1-Cb-com1	5'-CGCTGCCAAAGTATCATTAGCA-3'
Loop2-Cb-com1	5'-CGCTGCCAAAGTATCATTAGCAG-3'

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Bacterial Strains

The genome of *C. burnetii* (Nine Mile, ATCC 13032) was kindly gifted by the Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman (Kerman, Iran). Twelve bacterial strains were purchased from Microbial collection of Iran (Pasture Institute of Iran) and Persian Type Culture Collection (Iranian Research Organization for Science and Technology) (Table 2). The lyophilized stocks were revived in brainheart infusion broth (BHI) medium (Himedia, Germany) according to current laboratory methods. Genomic DNA was extracted by DNA Extraction kit (Sinaclon Company, Tehran, Iran) according to manufacturer's recommendations. Quality and quantity of the DNA templates were verified by agarose gel electrophoresis and spectrophotometrically at 260/280 nm, respectively. Confirming presence of amplifiable DNA in the extracted genomes, 16S rRNA gene was amplified with the broad range universal primers. The confirmed templates were kept at -20˚C till further use in specificity testing.

Table 2. Negative control bacteria used for specificity testing in this study

Organism	Strain
Shigella sonnei	ATCC 9290
Escherichia coli	ATCC 25922
Citrobacter freundii	PTCC 1600
Serratia marcesens	PTCC 1111
Salmonella typhi	PTCC 1609
Staphylococcus aureus	ATCC 25923
Bacillus subtilis	ATCC 6051
Yersinia enterocolitica	PTCC 1480
Proteus vulgaris	PTCC 1079
Enterococcus faecalis	ATCC 29212
Klebsiella pneumoniae	ATCC 7881
Pseudomonas aeruginosa	ATCC 27853

The LAMP assays

The LAMP reactions were performed in a total volume of 25 µL containing the following: 1µL of primer mix [5 pmol/µL of each outer primer (F3 and B3), 40 pmol/µL of each inner primer (FIP and BIP)], 12.5 µL of 2X reaction mix containing [10X ThermoPol reaction buffer (Biolabs, B9004S) containing [20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH4)_2$ SO_4, 0.1% Triton X-100], 2 mM $MgSO_4$ (AppliChem 10034-99-8), 0.2% Tween 20 (Acros, 233361000) (pH 8.8), 2.8 mM ofdeoxynucleotide triphosphate (dNTP), 1.6 M of betaine solution (Sigma, B030-1VL)], 1 µL (8U) of Bst DNA polymerase large fragments (New England Biolabs, Ipswich, MA, USA), 9.5 µL double distilled water and 2 µL of template genomic DNA. The mixture was incubated at 65˚C, 30 or 60 min (amplification) followed by heating to 80˚C for 5 min (enzyme inactivation) using a Loopamp real-time turbidimeter (LA-320C; Teramecs, Kyoto, Japan). To evaluate effect of the loop primers on increasing LAMP reaction rate, additional tests were carried out identically as described above

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except that 20 pmol/µL of each loop primers (LF and LB) were added to primer mix. Electrophoresis of the LAMP products on 2% agarose gel in addition to direct visual observation of turbidity by naked eye and inspection of amplification curve depicted by the Loopamp real-time turbidimeter were used concurrently to verify the LAMP reaction status in tubes (23).

Specificity Testing

To evaluate the test specificity, the LAMP reactions were optimized using genomic DNA of *C.burnetti* and genomic DNA of non-Coxiella organisms (Table 2). Reaction mix components, temperature program and analysis of LAMP products were same as aforementioned conditions. The experiment was performed in triplicate.

TA Cloning and Preparation of Standard Plasmid

A 197 bp fragment of the *com1* gene was PCR amplified using the LAMP F3 and B3 outer primers (Table 1). The PCR product was purified using a PCR purification Kit (Bioneer, Seoul, Korea). The purified fragment was ligated into the pTZ57R/T vector by 1U of the T4 DNA ligase. Then the *E. coli* JM107 competent cells were transformed by the ligation reaction product. All ligation, transformation and cloning process was performed according to manufacturer instructions of InsTAcloneTM PCR Cloning Kit (Fermentas). The recombinant clones on the solid medium were identified through blue/white screening and some of the white-colored colonies were selected for further evaluation. Afterwards, the selected clones were subjected to colony PCR using F3 and B3 outer primers. One of the PCR confirmed clones was selected for plasmids DNA extraction using AccuPrep Plasmid Mini Extraction kit (Bioneer, Seoul, Korea). The confirmed recombinant plasmid pTZ57R/T-*com1*, quantified by UV absorbance measurement at 260 nm and 280 nm quantified using a Picodrop spectrophotometer (Picodrop Limited, Saffron Walden, and UK). It was kept at -20˚C until used in sensitivity testing and quantitative assessment as the positive control.

Sensitivity and Quantification

A tenfold serial dilution of pTZ57R/T-*com1* plasmid (4-4×10^{-9} ng or 1.2×10^{9}-1.2 copies) was prepared. All dilutions were subjected to the optimized *com-1* LAMP assay in triplicate. Mixture of reaction, temperature program and detection methods of LAMP products were same as aforementioned above. The last dilution with an amplification related signal showed the test sensitivity or Limit of Detection (LOD) of the assay. Data acquired from the Loopamp real-time turbidimeter in sensitivity testing was used for launching a quantitative lamp (Q-LAMP). For this, a standard curve was depicted by plotting the Tt (Time threshold) values against log copy number of *com1* sequence and then linear regression was calculated.

Results

LAMP Setup

A white turbidity due to magnesium pyrophosphate precipitation was observed with the naked eye in the positive reaction tubes indicating amplification of *com1* gene. Analysis of the products by electrophoresis on 2% agarose gel demonstrated a clear ladder-like DNA pattern. Amplification graph of the Real-time turbidimeter showing an OD of 0.5 also confirmed amplification of the *com 1* gene. Adding the loop primers (LF and LB) to the LAMP reaction tubes reduced amplification-related generation time from 60 min to 30 min. Negative control tubes showed no signal in each of the detection methods in the experiments (data not shown).

Sensitivity, Quantification and Specificity

The lowest concentration of pTZ57R/T-*com1*

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detected by the *com-1* LAMP was determined in the sensitivity experiment. Using agarose gel electrophoresis to detect LAMP products, LOD was 0.04 fg or 12 copies of *com1* gene. Whereas the value was 0.4 or 120 copies when visual inspection with naked eye or using turbidimeter to track the LAMP products (Figure 1). In quantitative assays, a judgment graph related to evaluation of the serial dilutions was generated by the Real-time turbidimeter. For dilutions containing 12×10^6 to 12 copies of pTZ57R/T-*com1*, the average Tt value was 37 to 63 min respectively (Fig 1C). The standard curve was depicted by plotting each Tt value against the logarithm of copy number of pTZ57R/T-*com1*.

The constructed curve had a correlation coefficient (r^2) of 0.99. With this r^2, all points lied exactly on a straight line with no scatter (data not shown). Knowing Tt value of the sample let us predict copy number of the target gene perfectly. Specificity of the LAMP assay was proven when the Real-time turbidimeter judgment graph determined exclusive amplification for *com 1* gene, whereas was negative for the 11 non-Coxiella bacterial species. Furthermore, gel agarose electrophoresis of the LAMP products indicated that the ladder-like multi-band feature appeared only in tubes containing *Coxiella genome* DNA. Congruent with *in silico* analysis using BLAST, this finding revealed no false-positive or false-negative amplifications.

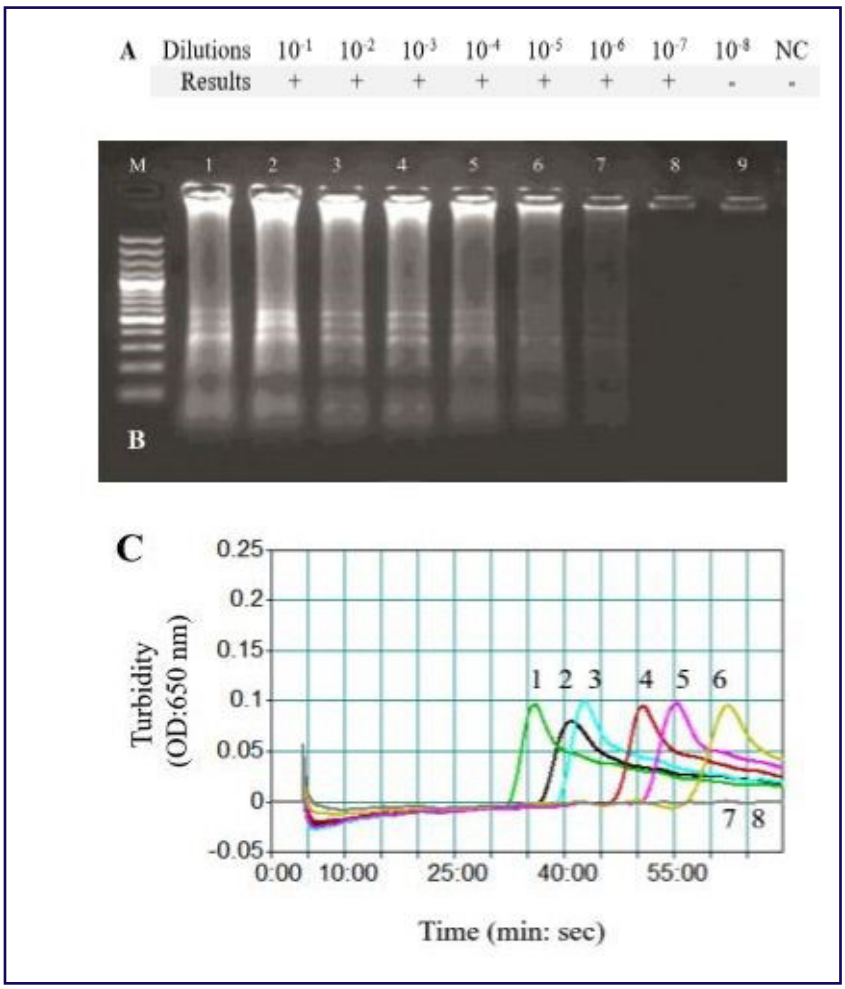


Figure 1. Sensitivity of the LAMP assay based on ten-fold serial dilutions of pTZ57R/T-*com1*. (A) Turbidity assay by naked eye observation. (B) Electrophoresis of the products showing ladder-like bands on the gel. Lane M, 100 bp DNA ladder; lanes 1–8, serial dilution of 10^{-1} to 10^{-8}; lane 9, negative control. (C) A typical turbidity judgment graph. Curves 1–8, serial dilution of 10^{-1} to 10^{-8}; curve 9, negative control

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Discussion

Currently, Q fever is considered as an emerging disease in various regions of the world.

Today, transmission of this microorganism through aerosol, the disease's getting epidemic, lack of human vaccine, unsafety of vaccine strains and *C.burnetii's* inclusion among the Biosafety Level 3 diseases (BSL3) have caused this microorganism to be used in bioterrorism.

In order to detect the *C.burnetii* bacteria, classical methods (sampling, cultivation of microorganism in the appropriate cell categories, such as (P388D1, J774 and L929), serological testing such as (immunofluorescence antibody, complement fixation test, Immunohistochemistry and ELISA) or molecular biology techniques such as (PCR, Nested PCR and Real Time PCR) can be used. Molecular techniques can be very effective because of their high precision and speed in the diagnostic process. However, these techniques have disadvantages including being costly, requiring special laboratory conditions and being time consuming. Therefore, localization of molecular techniques for the diagnosis of Q fever in the country is recommended. Now performing numerous studies around the world has been possible for detection of pathogens including bacteria, viruses and parasites by the LAMP technique. In Iran also, several studies have been conducted in connection with the LAMP technique, but no article has been published about using LAMP method to detect *C burnetii.* LAMP technique, despite its high precision and sensitivity, does not need advanced laboratory equipment and the reaction is conducted using a simple turbidimeter device. The present study describes the *com1* LAMP assay, a quantitative molecular method that is more rapid, sensitive, and cost-effective than other methods of DNA amplification used for laboratory diagnosis of the organism. Different sequences of the of *C. burnetti* genome has been used in its detection, including 16S *rRNA*, *ISIIII*, *ISIIIIa*, *Sod B*, *Icd, glt*, *htpA*, *htpB*, *omp*, *pyr B*, *qrsA*, *DNA j*, *muc Z, SERS*, *algC* and the 16S-23S intergenic spacer region (24). Our assay was developed based on targeting *com1* because this gene is highly conserved in *C. burnetii* and is present in a single copy which allows more accurate quantification of the pathogen load in the samples (25). No special processes are needed for the LAMP assay, which can be determined by the naked eye, in contrast to electrophoresis. In addition, *Bst* DNA polymerase enzyme shows high resistance to inhibitory factors in the amplification reaction. These features make it valuable to serve in low-resource settings and large scale use (e.g., epidemiological studies) in laboratories with limited equipment (26). The primers designed in this study were specific to the *com1* gene and amplification was performed only in presence of *Coxiella* DNA hinting analytical specificity of 100%. Our results confirmed that application of the loop primers that attach to the stem loops formed during the reaction process enhanced amplification speed and efficiency as previously described by Nagamine (27). A total time of nearly 60 min was required for the LAMP assay, including amplification and detection. However, use of the loop primer decreased the working time to 30 min. No cross-amplification was detected in the negative control samples so the assay had complete specificity to detect trace amounts of *Coxiella* DNA. The high specificity could result from the use of the six primers that recognize the distinct regions on the target sequences (28, 29). Sensitivity of the *com1* LAMP when using visual observation of turbidity or the Real-time turbidimeter and gel agarose electrophoresis as detection methods was 120 and 12 copies of *com1* gene, respectively. Howe et al, performed sensitivity test through PCR test. They prepared 10 dilution serials from concentration of 10pg-10fg and obtained reportable detection limit of 50 for gene *com1* and 57 for *IS1111* (29). Klee et al, established Real-time PCR Test based on TaqMan. This test was done targeting two markers of *Icd* and transpose from *IS1111a* in multiple copies in the genome of *C. burnetii*. The results showed that minimum number of genomes that can be detected in each reaction is 10 *Icd* markers and

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6 IS marker (29). Ito et al, did rapid detection and isolation of influenza virus type A and B conducted by the LAMP technique and compared this technique with immunochromatography. LAMP Primer compounds for influenza type 1 (HA1) and type 3 (HA3) and type B influenza viruses had higher sensitivity than those with commercially available and rapid diagnostic tests (30).Imai et al, launched the H5-RT-LAMP system for rapid detection of influenza H5 in birds. LAMP Primer based on Hemagglutinin can detect H5N1 influenza virus with sensitivity 100 times higher of RT-PCR (31). Pan et al, was the first study to diagnose *Coxiella* using the LAMP assay. They used the htpAB gene and suggested that this approach was a suitable alternative for PCR and real-time PCR assays. The limit of detection for their assay was 1 copy per reaction (32). The real-time LAMP assay in the present study was able to detect 120 copies of *Coxiella* DNA in a reaction tube, which is comparable to detection limits achieved by other molecular methods such as real-time PCR (33). In our quantitative real time LAMP, the standard curve depicted by plotting the Tt values against log copy number of pTZ57R/T-*com1* had a correlation coefficient (r^2) of 0.99 and also the standard curve showed a linear range of 0.04µg-0.04fg (12×10^6 to 12) for pTZ57R/T-*com1*. So, the assay is suitable to quantify *C. burnetii* in un-known samples in the above ranges. However, evaluation of the assay by using samples from patients or artificially contaminated materials is required.

A disadvantage of LAMP is its susceptibility to cross-contamination, that is, the material in the aerosol (34). As a result, it is recombined to allow rooms to be ventilated and different samples to be analyzed separately. For obvious reactions, this may not always be possible. Another disadvantage of LAMP is that examination of samples varies in terms of the presence of reaction inhibitors, as this requires two reactions, one to see the inhibitors and the other to strengthen the ingredients.

Conclusions

The simplicity, low cost (3- to 6-fold less than PCR and real-time PCR), and having no need for expensive and complex equipment make the LAMP method preferable to other molecular methods. It is also highly sensitive and specific to *Coxiella* quantitative detection. This method could be beneficial for the rapid detection of *Coxiella* infections in humans and domestic animals in resource-limited settings in developing countries which could be of importance in epidemiological studies.

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Conflict of Interest

The authors have no conflict of interests.

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Code of Ethics

This study is financially supported by Islamic Azad University, QOM Branch in 2019 code: 97800.

Data Availability Statement

All data related to this research have been included in the manuscript.

Authors' Contributions

M. Soleimani is the corresponding author, performed the laboratory tests, drafted the manuscript, and participated in data analysis. K. Majidzadeh-A designed and supervised the study, analyzed and interpreted the data, and reviewed the manuscript. Razieh Zabihi and Abbas Morovvati participated in laboratory work and contributed to data analysis. All authors read and approved the final version of the manuscript.

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