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Development of loop-mediated isothermal amplification assays for rapid and easy detection of *Coxiella Burnetii*

Hua-Wei Chen [a,b], Wei-Mei Ching [a,b,*]

[a] Naval Medical Research Center, Silver Spring, MD, United States
[b] Uniformed Services University of the Health Sciences, Bethesda, MD, United States

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ABSTRACT

Q fever is an important worldwide zoonosis that is caused by infection with *Coxiella burnetii*. We have developed a loop-mediated isothermal amplification (LAMP) assay to detect the presence of the transposase gene insertion element *IS1111a* of *C. burnetii*. The sensitivity of this LAMP assay is very similar to quantitative PCR (qPCR) method with a detection limit at 25 copies of the gene, the equivalent of about one *C. burnetii* organism. Several methods for the detection of LAMP product were also performed to show the diverse way of detection which may be used in different settings depending on the user's infrastructure and resource.

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1. Introduction

Coxiella burnetii, a small Gram-negative bacterium is the causative agent of Q fever which is a worldwide zoonosis. Due to Q fever's worldwide distribution and the high infectivity of *C. burnetii*, the US military and civilian personnel deployed overseas are at high risk of being infected.

Q fever manifests in two forms: acute and chronic infections. The acute Q fever illness most commonly presents as a flu-like illness, pneumonia, or hepatitis; however, asymptomatic infections may occur. Symptoms of Q fever are easily confused with those due to a variety of other pathogens (e.g., dengue and malaria) that may require different treatment regimens. The chronic form of the disease is infrequent (<5% of patients with acute infection), but the potentially consequent endocarditis is often fatal if left untreated (Rolain et al., 2005; Anderson et al., 2013). Therefore, early diagnosis to guide an appropriate treatment is critical for patient care. Detection and quantification of the bacteria by conventional culturing methods are time consuming and dangerous. PCR based diagnostic assays have been developed for detecting *C. burnetii* DNA in cell cultures and clinical samples (Fournier and Raoult, 2003; Turra et al., 2006; Schneeberger et al., 2010). Because PCR method requires specialized equipment and extensive end user training, it is not suitable in resource-constrained areas for routine work.

Loop-mediated isothermal amplification (LAMP) assay is a rapid DNA amplification method originally developed by Notomi et al (2000). It has been applied for the detection of several rickettsial pathogens (Paris et al., 2008; Huber et al., 2012; Pan et al., 2013). The method requires a specially designed primer set that recognizes at least six independent regions of the target gene, which increases the specificity as well as the rapidity of the reaction. Since the *Bst* DNA polymerase used in LAMP allows strand displacement-DNA synthesis, it is an autocycling strand displacement DNA synthesis method that can be performed at a single temperature around 60°–65 °C. LAMP reactions are performed under isothermal conditions using a simple incubator, such as a water bath or a heating block. The results are visualized by turbidity that can be seen by the naked eye (Mori et al., 2001), and optionally by agarose gel electrophoresis or by addition of fluorescent dyes to be visualized under UV light (Qiao et al., 2007; Tomita et al., 2008).

We have developed a loop-mediated isothermal amplification assay to detect the presence of *C. burnetii* in plasma. Five sets of primers were designed using the transposase gene insertion element *IS1111a*. The amplification reaction mixtures were incubated at 60 °C for 60 min. We were able to detect about 25 copies of bacterial DNA (an equivalent of one organism) in the reaction using DNA extracted from human plasma samples spiked with either DNA plasmid containing the *IS1111a* or *C. burnetii* genomic DNA. The sensitivity of the LAMP assay was similar to qPCR. In this study, we also included SYBR green or hydroxy naphthol blue (HNB) in the reaction mixture for product detection. In addition specially labeled primers combined with immune-chromatography test (ICT) provided another easy-to-use detection system. Our results suggest that this assay has the potential to be used as a rapid, robust, and easy-to-perform assay in the endemic regions.

* Corresponding author at: Naval Medical Research Center, 503 Robert Grant Ave, Silver Spring, MD 20910, United States. Tel.: +1 301 319 7438; fax: +1 301 319 7451.
E-mail address: weimei.ching@med.navy.mil (W.-M. Ching).

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2. Materials and methods

2.1. Design of primers

Oligonucleotide primers used for the LAMP and qPCR assays were designed based on the transposase gene insertion element *IS1111a* of *C. burnetii* RSA 493. Five sets of primers were designed using PrimerExplorer V4 (http://primerexplorer.jp/e/). The primers used for the qPCR assay were previously described (Klee et al., 2006). All primers were synthesized by Eurofins MWG Operon (Huntsville, AL) and are described in Table 1.

2.2. Plasmid and genomic DNA template

The gene *IS1111a* of *C. burnetii* RSA 493 was cloned into a pET24a vector, and the closed circular plasmid (pET24a-IS1111a) was purified using standard Qiagen plasmid mini kit (Qiagen, Stockach, Germany) following the manufacturer's instruction. The pure pET24a-IS1111a was quantified using a Nano-drop 2000 microsample spectrophotometer (Thermo Scientific, Wilmington, DE) and used as a standard in the selection of the best primer combination used in the LAMP assay. The genomic DNA from *C. burnetii* RSA 493 was used as template in both LAMP and qPCR as described below. The genomic DNA from other phylogenetically closed bacteria (*Orientia tsutsugamushi*, *Rickettsia typhi*, *Rickettsia conorii*, *and Rickettsia rickettsi*) were used in LAMP to evaluate the assay's specificity.

2.3. LAMP reaction

LAMP reactions were carried out as described previously (Notomi et al., 2000). Briefly, a 25 μl reaction mixture contained 1.6 mM of each FIP and BIP primer, 0.8 mM of each LF and LB primer, 0.2 mM of each F3 and B3 primer, 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.8 M betaine (Sigma-Aldrich, St. Louis, MO), 1.4 mM dNTP mixture (New England Biolabs, Beverly, MA), 8 U *Bst* DNA polymerase (New England Biolabs, Beverly, MA), and DNA template. The reaction mixture was incubated at 60 °C for 60 min. Each reaction was terminated by adding 5 μl of 10X BlueJuice (Invitrogen, Carlsbad, CA) for gel detection. The reaction products were examined by electrophoresis on 2% agarose gel stained with a 1:10,000 dilution of GelRed (Phenix Research Products, Asheville, NC) and visualized by UV light. Other detection methods, such as inclusion of HNB (Wastling et al., 2010) into the reaction mixture was used to visualize the reaction results with naked eyes or inclusion of SYBR green was used to detect the reaction products by a UV light. Furthermore, the inclusion of SYBR green in the reaction mixture also allowed real-time detection with fluorescence measurement systems such as ESEQuant Tube Scanner (Qiagen, Stockach, Germany) and 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA). All detection assays were performed in triplicate.

Table 1
List of primer sequences for five LAMP primer sets and qPCR primers (5′-3′).

QF1-F3	GACGGGTTAAGCGTGCTC
QF1-B3	CTGCGCATCGTTACGATCA
QF1-FIP	GCTCCTCCACACGCTTCCATTGTATCCACCGTAGCCAGTC
QF1-BIP	ATCGGACGTTTATGGGGATGGGACATACGGTTTGACGTGCTG
QF2-F3	CGTAGCCAGTCTTAAGGTGG
QF2-B3	GCGCTTGAACGTCTTGTTG
QF2-FIP	GGACTGATCAACTGCGTTGGGAGTGTGGAGGAGCGAACCA
QF2-BIP	CGTAACGATGCGCAGGCGATTTACCCTGCACAAACCGC
QF2-LF	ACCCATCCCCATAAACGTCCGA
QF2-LB	AGCTGAAGCGGCTTCCC
QF3-F3	GTGGCAAAAGCCAATGAGG
QF3-B3	CCGCGTTTACTAATCCCCAA
QF3-FIP	GCATAAACCGAGAGCGCCGTTATTGTCAACGGGTACAGAGC
QF3-BIP	TCATCGTTCCCGGCAGTTGTCCACCTCCTTATTCCCACTCG
QF3-LB	GGGTTGGTCCCTCGACAACAT
QF4-F3	CGGATGAAACGGGTGTTGAA
QF4-B3	AACTGCCGGGAACGATGA
QF4-FIP	TTGGCTTTTGCCACCGCTTTTGAATTGTTGAACCGGGACGA
QF4-BIP	GTACAGAGCATCCCGGGGGTTCACCCACGCTCGCATAA
QF5-F3	GGACGAAGCGATTGGTGATT
QF5-B3	TTCCCACTCGAATGTTGTCG
QF5-FIP	CGTTAAATAACCCACCCCCGGGGTGGCAAAAGCCAATGAGG
QF5-BIP	CGCTCTCGGTTTATGCGAGCGAACCCAATAAACGCCGACA
QF5-LB	GGGTGACATTCATCAATTTCATCGT
QF	GTCTTAAGGTGGGCTGCGTG
QR	CCCCGAATCTCATTGATCAGC

2.4. Sensitivity of the LAMP assay

The serial dilutions of pET24a-IS1111a plasmid were used to determine the limit of detection. Quantitative PCR performed on 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA) was used to confirm the sensitivity of the LAMP assay. Primers designed against *IS1111a* gene sequence described in previous study were used (Klee et al., 2006) (Table 1). The total volume of each reaction was 20 μl. Each reaction mixture contained 0.5 μM of forward primer, 0.5 μM of reverse primer, 1X RT2 SYBR Green qPCR Mastermix (SA-Biosciences, Frederick, MD) and DNA template. An initial 10 minute activation step at 95 °C was followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min.

2.5. Specificity of the LAMP assay

Genomic DNA from three major *Rickettsia* species (*R. typhi*, *R. conorii*, and *R. rickettsii*) and four strains of *O. tsutsugamushi* (Karp, Kato, Gilliam, and TA763) were used to verify the specificity of the assay.

2.6. Feasibility of LAMP assay for plasma samples

Normal human plasma (SeraCare, Milford, MA) spiked with pET24a-IS1111a plasmid or *C. burnetii* genomic DNA were subjected to DNA extraction using QIAamp DNA Mini Kit (Qiagen, Stockach, Germany) according to the manufacturer's manual. A total of 200 μl plasma samples were used for extraction and extracted DNA were eluted in 20 μl volume. Three DNA extractions were performed independently.

2.7. Immuno-chromatography (ICT) strip cassette for amplicon detection

Type II BESt Cassettes (BioHelix, Beverly, MA) were used to detect LAMP products in an instrument-free and cross-contamination-proof manner. These cassettes were capable to detect an amplicon that is dual labeled with biotin and fluorescein. BESt cassette consists of two parts: an inner amplicon cartridge and an outer detection chamber. The amplicon cartridge holds running buffer and a reaction tube in place and the detection chamber holds a DNA test strip. We used 5′-biotin-labeled QF3-FIP primers and 5′-FAM-labeled QF3-LB primers in the LAMP reactions in a 0.2 ml PCR tube. After amplification reaction, the reaction tube was inserted in an amplicon cartridge next to running buffer and the cassette was closed. The results were read by the presence or absence of the test line after 10 min at room temperature.

3. Results

3.1. Selection of the best primer set

The LAMP reactions were performed under isothermal conditions in a range of 58 to 63 °C using pET24a-IS1111a plasmid for 60 min. Among those five sets of primer, primer set-3 performed the best, and it detected 100 copies per reaction at 60 °C (Fig. 1). Primer set-4 did not work at all (not shown). Therefore, the optimized temperature of 60 °C and primer set-3 were used for the rest of experiments.