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Evaluation of the stability of lyophilized loop-mediated isothermal amplification reagents for the detection of *Coxiella burnetii*

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Abstract

Coxiella burnetii, the causative pathogen for Q fever, is an obligate intracellular bacterium and designated as a biosafety level 3 agent. Detection and quantification of the bacteria with conventional culturing methods is time-consuming and poses significant health risks. Polymerase chain reaction (PCR)-based assays have been developed for detecting *C. burnetii* and could provide rapid diagnosis. However, they require specialized equipment, including a cold chain for PCR reagents that maintains their stability during storage and transport. These requirements limit the advantage of PCR-based methods, especially in resource-limited areas.

Previously, we had developed a lyophilized loop-mediated isothermal amplification (LAMP) assay to detect the presence of *C. burnetii*. To simplify and improve this assay, the reagents for the LAMP assay and the detecting reagent, SYBR green, were lyophilized together. The stability of the lyophilized reagents was evaluated by measuring changes in detection limit for plasmid DNA encoding a *C. burnetii* gene upon storage at 4 °C, 25 °C, or 37 °C. Our data indicate that the lyophilized reagents remain stable for 24 months when stored at 4 °C, 28 days at 25 °C, and 2 days at 37 °C. This improved LAMP assay can be easily performed in a simple water bath or heating block. The stability at ambient temperature, the

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simplicity of assay procedure, and the availability of low cost equipment make this method ideal for use in resource-limited settings where Q fever is endemic.

Keywords: Biotechnology, Microbiology, Infectious disease

1. Introduction

Q fever is a worldwide zoonotic disease caused by the infection with *Coxiella burnetii*. Most human cases are believed to come from the inhalation of windborne dust contaminated with *C. burnetii*. Contamination stems from the reproductive tissues of domestic livestock, primarily sheep, goats, and cattle [1, 2]. Domestic pets, wild animals and ticks are also important reservoirs [1, 2]. Among them, kangaroos have been identified as possible reservoirs in the transmission of *C. burnetii* and the acquisition of Q fever in Australia [3]. Disease has also been associated with consumption of unpasteurized milk and tick bites [4].

Earlier studies showed that Q fever poses a greater threat to US forces deployed in Iraq than previously predicted [5, 6, 7, 8]. A febrile illness outbreak investigation among marines in Iraq found that Q fever is capable of causing localized outbreaks in exposed personnel, with attack rates up to 50% and higher [8]. As a result, the U. S. Army Center for Health Promotion and Preventive Medicine (USACHPPM) initiated a Q fever surveillance program in early 2007. There have been more than 150 confirmed cases reported among U.S. military personnel deployed to Iraq since 2007 [9]. In addition, the Netherlands had a large outbreak of Q fever from 2007 to 2010 with over 4,000 human cases, making it the largest Q fever outbreak ever reported [10].

Acute Q fever most commonly presents as a flu-like illness, hepatitis, or pneumonia, and is usually a self-limiting disease with a low mortality rate [1, 11]. Chronic Q fever is less frequent but has higher potential to progress and present as endocarditis, which has a higher mortality rate if left untreated [12, 13]. Therefore, early diagnosis is essential to guide an appropriate antibiotic therapy for patient care. Polymerase chain reaction (PCR) based diagnostic assays have been developed for detecting the genomic DNA of *C. burnetii* in cell cultures and clinical samples [14, 15, 16]. However, these assays require expensive equipment and experienced end users, both of which are often not readily available in resource-limited areas for routine work.

Originally described by Notomi et al., loop-mediated isothermal amplification (LAMP) is a DNA amplification method that uses 2–3 pairs of sequence-specific primers and a DNA strand-displacement process for amplification under isothermal conditions [17]. This method has been used to detect several rickettsial pathogens [18, 19, 20]. Recent reports described successful detection of pathogens from minimally processed samples, such as heat-treated blood, to a wide range of

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sample matrices such as stool, soil and milk [21, 22, 23, 24, 25, 26]. The advantage of LAMP is that amplification occurs under isothermal conditions. Therefore, only a simple incubator, such as a water bath or a heating block, is sufficient to perform the reaction. The amplified products can be visualized by agarose gel electrophoresis, using a fluorescent dye such as SYBR green to be visualized under UV light or by hydroxy naphthol blue: a metal indicator that can be seen by the naked eye [27, 28].

We have previously developed a highly specific and sensitive LAMP assay to detect the presence of *C. burnetii* in plasma samples. The limit of detection for this LAMP assay is about 25 copies of the *IS1111a* gene which is equivalent to one *C. burnetii* organism [29]. The reagents for the LAMP assay were lyophilized. A comparison between the lyophilized reagents and non-lyophilized reagents showed comparable sensitivity [30]. In the present study, the stability of the lyophilized reagents with the detecting reagent, SYBR green, was evaluated following storage at 4 °C, 25 °C, and 37 °C to determine the feasibility of eliminating cold chain storage.

2. Materials and methods

2.1. Primers

The primer sets used in this study were the same as the ones described previously in Chen et al. [29, 30]. All primers were synthesized by Eurofins MWG Operon (Huntsville, AL); FIP and BIP primers were HPLC purified.

2.2. DNA template

Element *IS1111a* of *C. burnetii* RSA 493 was cloned into vector pET24a to make an *IS1111a* target DNA template. The purified pET24-IS1111a was quantified using a Nano-drop 2000 microsample spectrophotometer (Thermo Scientific, Wilmington, DE) and used as a standard in the LAMP assay to determine the assay's sensitivity as described previously [29, 30].

2.3. Lyophilized reagents

Bst DNA polymerase (8 U, glycerol-free), SYBR green (1x), dNTPs (1.4 mM), and primers (0.2 µM each of F3 and B3, 1.6 µM each of FIP and BIP, and 0.8 µM of LB) were supplemented with sugars and subjected to lyophilization in a freeze-dryer using a protocol based on the method described by Saleki-Gerhardt and Zografi [31]. This service is provided by GeneReach USA (Lexington, MA) [30]. Vials containing those reagents were packaged in a zipped aluminum foil bag (Fig. 1).

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Fig. 1. Lyophilized LAMP reagents for the detection of *Coxiella burnetii*. The lyophilized reagents were inside a 0.2 ml vial containing *Bst* DNA polymerase, SYBR green, primers, and dNTPs. Vials were packaged in a zipped aluminum foil bag.

2.4. Storage of lyophilized reagents at different temperatures

Lyophilized reagents were stored inside the zipped aluminum foil bags at 4 °C, 25 °C, and 37 °C. Four degrees Celsius is the suggested storage temperature by GeneReach. Twenty five degrees Celsius was selected to mimic normal room temperature, and 37 °C was selected to represent high heat conditions. At selected time points from day zero to 720 days (Table 1), the lyophilized reagents were reconstituted with reconstitution buffer for LAMP reactions.

Table 1. The detection limit (copy number) of a loop-mediated isothermal amplification (LAMP) reaction to detect *Coxiella burnetii* using lyophilized reagents stored at 4 °C, 25 °C, and 37 °C[a] for 0–720 days.

Storage (days)	0	2	7	14	28	42
4 °C	25[b]	25	25	25	25	25
25 °C	25	NA[c]	25	25	25	50
37 °C	25	25	50	100	100	100

Storage (days)	60	90	180	270	360	450	540	630	720
4 °C	25	25	25	25	25	25	25	25	25
25 °C	50	50	50	50	100	100	100	NA	NA

[a] Reaction mixtures were incubated at 60 °C for 60 min.
[b] Numbers were the amount of pET24-IS1111a plasmid copies in the reaction.
[c] NA: did not test.

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2.5. Reaction mixture for lyophilized reagents

For each reaction mixture, 20 μl of reconstitution buffer containing 25 mM Tris-HCl (pH 8.8), 12.5 mM KCl, 10 mM $MgSO_4$, 12.5 mM $(NH_4)_2SO_4$, 0.125% Triton X–100, 1 M betaine was added into the vial with lyophilized reagents as previously described [30]. Serial dilutions of 20, 10, and 5 cp/μl of pET24-IS1111a plasmid were prepared. Lastly, 5 μl of DNA template was added to start the reaction.

2.6. LAMP reaction

The reactions were carried out as described previously [29, 30]. In brief, a 25 μl reaction mixture contained 1.6 mM of each FIP and BIP primer, 0.8 mM of 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, Ipswich, MA), 8 U *Bst* DNA polymerase (New England Biolabs, Ipswich, MA), and 5 μl of DNA template. The reaction mixture was incubated at 60 °C for 60 min. The LAMP amplified DNA 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 using UV light. Since the detecting reagent SYBR green was present in the lyophilized reagents, the results were visualized directly from the reaction vial with a UV lamp. At each storage time point, the assays were performed at 100, 50, 25, and no copies of pET24-IS1111a plasmid in duplicate.

3. Results

First, the LAMP reactions were performed using both the non-lyophilized reagents and lyophilized reagents. The LAMP reaction results on agarose gels showed that both reagents can detect 25 copies of DNA template (Fig. 2). The sensitivity of these lyophilized reagents remains at 25 copies; similar to the non-lyophilized reagents and consistent with our previous results [29, 30].

The stability of the lyophilized reagents was evaluated in the reactions using the lyophilized reagents at different time points after storage at 4 °C, 25 °C, and 37 °C. The duration of stability of the lyophilized reagents without losing any sensitivity was 720 days, 28 days, and 2 days at 4 °C, 25 °C, and 37 °C, respectively (Table 1).

4. Discussion

The availability of a cost-effective, rapid nucleic acid detection test could potentially improve detection of acute infection at point-of-care or in resource-limited settings. LAMP represents an attractive approach for rapid and deployable pathogen detection. Previously, we developed a sensitive and specific LAMP assay

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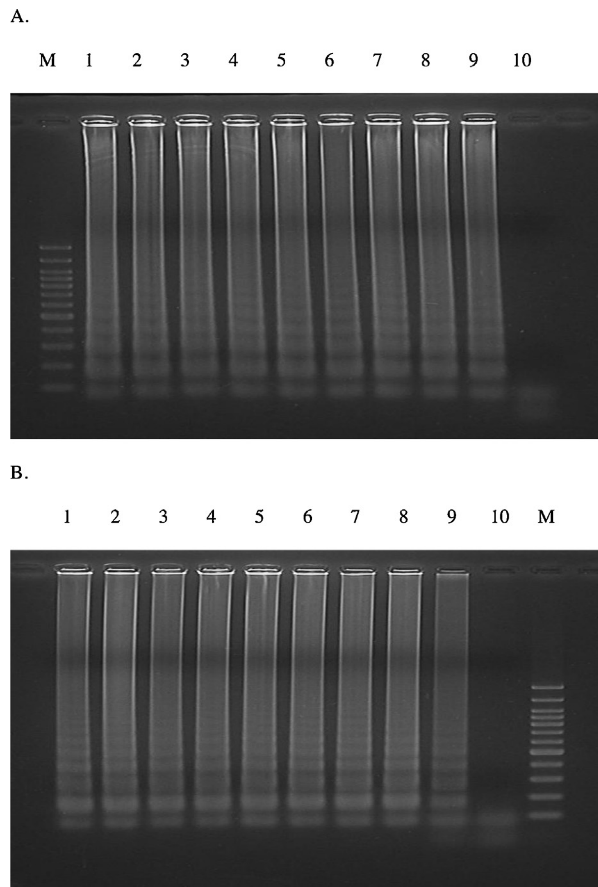

Fig. 2. LAMP results on agarose gel with non-lyophilized reagents (A) and lyophilized reagents (B) for the detection of *Coxiella burnetii*. Reaction mixtures were incubated at 60 °C for 60 min. Lane M, 100 bp ladder; Lane 1–3, 4–6, 7–9 and 10 are reactions with 100, 50, 25, and no copies of pET24-IS1111a plasmid.

targeting the insertion element *IS1111a* [29]. The *IS1111* element was selected because of its high conservation of gene sequence among different strains of *C. burnetii* and its high number of copies (7 to 110) in the bacteria [32]. Our results showed this LAMP assay can detect about 25 copies of the *IS1111* element, the equivalent of as little as one chromosomal copy of *Coxiella* DNA. In this study, we reported a LAMP assay for detection of *C. burnetii* that addressed some of the major limitations of LAMP which had hindered its deployment in resource-limited settings. With the inclusion of SYBR green in the lyophilized reagents, the results of the LAMP reaction can be visualized with a UV light without running on an agarose gel. This eliminates the need to open the reaction vials after the LAMP reaction, simplifying the workflow and minimizing the risks of contamination. For better visibility and documentation of results, a transilluminator was used to visualize the reaction vials in this study. However, a portable, battery-operated fluorescent reader or handheld UV light can be used at point-of-care sites.

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Three temperatures (4 °C, 25 °C, and 37 °C) were chosen for the storage of lyophilized reagents. As the storage temperature increased, the duration of stability decreased. The reactions prepared from the lyophilized reagents stored at 4 °C for 720 days still showed a strong fluorescence signal with UV light when 25, 50, and 100 copies of DNA templates were present (Fig. 3A). The reactions prepared from the lyophilized reagents stored at 25 °C for 540 days only showed positive signal when 100 copies of DNA templates were present (Fig. 3B). Long term stability was observed at 4 °C with no loss of performance in detection limit for two years. Although the storage stability of lyophilized reagents was transient at 25 °C and 37 °C, a satisfactory performance (detection limit of 100 copies) was observed when the reagents were stored for up to 540 days and 42 days, respectively. These results suggested that the lyophilized LAMP reagents can be transported by normal post mailing without dry-ice protection and stored in places where even the refrigerators are not available. In resource-limited settings, an air-conditioned room which can maintain temperature around 25 °C will be a good place for long term storage of these lyophilized reagents. In the tropical locations where the temperature is constantly around upper thirties Celsius, the lyophilized reagents can still be useful after 42 days. All of these demonstrated the potential usage of LAMP in resource-

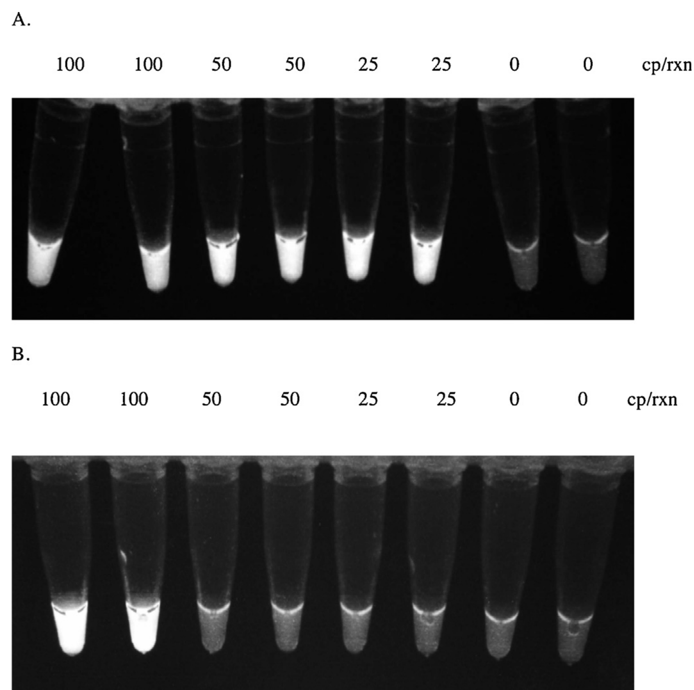

Fig. 3. Determine the detection limit of the lyophilized LAMP reagents for the detection of *Coxiella burnetii*. The lyophilized reagents were stored at 4 °C for 720 days (A) or 25 °C for 540 days (B) before reconstituted for reactions. Reaction mixtures were incubated at 60 °C for 60 min containing 100, 50, 25, and no copies of pET24-IS1111a plasmid.

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limited settings where the cold storage of reaction components might not be available.

A number of lyophilized LAMP and RT-LAMP assays have been developed for detection of bacteria, virus and parasites [33, 34, 35, 36, 37]. Carter et al. showed that the lyophilized reagent mixture remained stable at room temperature with little loss of activity for over one and half months [36]. Curtis et al. reported stability of the lyophilized reagents following storage at −20, 4, 25 and 30 °C for up to one month [34]. Our results were very similar with their findings, showing no loss of sensitivity after storage at 4 °C and 25 °C for one month.

Unlike PCR-based methods where a thermocycler is needed, the LAMP assay only requires a heating block or water bath that can maintain a temperature around 60 °C. Not only is the cost of a water bath or heating block is cheaper than a thermal cycler, but also the operation of a water bath or heating block is much simpler. Furthermore, a water bath/heating block is already available in many of the resource-limited settings where the LAMP assay is going to be implemented. The reagent cost of PCR is about $1 to $2 per reaction which is very similar to that of the LAMP assay at about $1.5 per reaction. Since there is no need for cold chains of storage and transportation on these lyophilized LAMP reagents, this makes the format even more affordable for resource-limited areas. For a regular LAMP assay, the DNA polymerase, primers, and dNTPs need to be stored at −20 °C. In contrast, the lyophilized reagents for the improved LAMP assay can be stored and transported long term at 4 °C and 25 °C. These improvements of accessibility, operation, and maintenance ultimately make this new method more useful for detecting *C. burnetii* in resource-limited settings where Q fever is endemic.

Declarations

Author contribution statement

Hua-Wei Chen: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Wei-Mei Ching: Conceived and designed the experiments; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

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Additional information

No additional information is available for this paper.

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