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Original Article

Development and validation of a rapid and simple fluorescence real-time loop-mediated isothermal amplification assay for detection of *Burkholderia pseudomallei*

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Abstract: Background and objective: Melioidosis is a disease caused by Burkholderia pseudomallei, which is a Gram-negative bacterium found in the environment that is distributed across the warm tropical regions. In these warm tropical regions, severe community-acquired sepsis caused by Burkholderia pseudomallei is relatively difficult to differentiate from other causes of sepsis, and rapid detection is required for prompt diagnosis and treatment. The aim of this study was to develop and evaluate a low-cost and effective method to detect Burkholderia pseudomallei in developing tropical countries. Methods: DNA extracted from the Burkholderia pseudomallei strain was isolated and used as the template in a loop-mediated isothermal amplification (LAMP) assay performed in a fluorescence real-time PCR analyzer. The amplified TTSS1-orf2 gene sequence was compared with the sequence in the National Center for Biotechnology database and used to design highly specific primers. Results: The LAMP method detected as little as 0.65 pg/μL of Burkholderia pseudomallei DNA, which is equivalent to 10^3 cfu/mL. In addition, the LAMP assay results had remarkable specificity and stability. Conclusion: LAMP is a sensitive and reliable method for detection of TTSS1-orf2 as a low cost, rapid, and valid Burkholderia pseudomallei detection method.

Keywords: Melioidosis, Burkholderia pseudomallei, LAMP, detection, TTSS1-orf2

Introduction

Melioidosis is a disease caused by Burkholderia pseudomallei, which is a Gram-negative bacterium exist in the environment commonly found in Southeast Asia and northern Australia where it causes severe community-acquired sepsis [1, 2]. Manifestations of melioidosis are highly variable, and melioidosis infections cannot be distinguished from other causes of sepsis on the basis of clinical features alone [3]. Culturing bacteria have been the golden standard in microbiological diagnoses, but isolation and identification of cultures takes a week, and melioidosis patients can deteriorate rapidly in one week [4]. Molecular detection technologies are advantageous because they are highly sensitive and fast [5], but PCR methods may be inappropriate for clinical examinations because PCR equipment is expensive, especially for hospitals in many developing countries [6, 7].

Loop mediated isothermal amplification (LAMP) proposed under 2000 and utilizes Bst DNA polymerase and 3 primer pairs that target 6 unique sequences in a DNA template amplifies with high specificity, efficiency and rapidity under isothermal conditions [8-10]. Although LAMP sensitivity and specificity are similar to traditional PCR [11], LAMP only requires a water

bath for amplification, and a DNA template can be amplified to 10^{10} copies in 35 to 60 minutes in a 65°C water bath [12]. Amplification can be identified easily by various methods, including direct visual observation of turbidity and turbidimetric measurements [13, 14].

The specificity of molecular diagnostic technologies is highly dependent on the target genes and the gene primers utilized. Several Burkholderia pseudomallei target genes have been used for PCR-based diagnostics, including 16S rRNA, lpx0, 8653, mprA, 266152, and TTSS1-orf11. TTSS1-orf2 is specific and conserved among various species of Burkholderia pseudomallei, and detection of TTSS1-orf2 has been shown to be highly sensitive [15, 16].

LAMP has become a popular method to diagnose numerous infectious diseases because of its relative simplicity and low cost, especially in developing countries [14]. In this study, we evaluated the efficiency of LAMP to detect Burkholderia pseudomallei by targeting TTSS1-orf2. We found that LAMP is a simple, low-cost, and rapid method to diagnose melioidosis, especially in humid regions of developing countries.

Methods

Clinical sample collection and DNA extraction

Nucleic acids from Burkholderia pseudomallei were provided by Hainan hospital. Clinical samples were mainly collected from the Third affiliated Hospital of Guangzhou Medical University in Guangzhou, southern China between March 2016 and December 2016. Sputum samples were harvested from patients using sterile swabs, and each sample was cultured on blood plates (Beiruite Bio-technology. Zhengzhou, China) and incubated for 48 hours at 36°C with 5% CO_2. Colony morphology was analyzed and cellular morphology was assessed by Gram-staining and microscopy. Bacterial DNA was isolated with a DNA isolation kit (Guangzhou Deaou Biotechnology) according to the manufacturer's instructions and was stored at -20°C until further use.

Fluorescent LAMP reaction

The sequence of Burkholderia pseudomallei TTSS1-orf2 was obtained from GenBank. The primers used in this study were developed by Primer Explorer version 4 online software (http://primerexplorer.jp/e) and were synthesized by Invitrogen, Guangzhou.

The LAMP mix without DNA templates was established on the ice, and then 2 μL of DNA template was added to 23 μL of the LAMP mix. The reaction mixture contained 1.6 μM each of the forward inner primer (FIP) and the backward inner primer (BIP), 0.2 μM each of the F3 primer and the B3 primer, 0.8 μM each of the loop forward (LF) primer and the loop backward (LB) primer, 40 mm Tris-HCl PH 8.8, 20 mm KCl, 16 mm $MgSO_4$, 20 mm (NH_4SO_4, 0.2% Tween-20, 0.8M Betaine) 22.8 mm of dNTPs each), 0.5 mL of a 1:100 dilution SYBR green I (Invitrogen), and 8 units of Bst polymerase (New England Biolabs, Ipswich, MA). The final mixture was amplified for 60 minutes at 63°C using an automatic real-time PCR analyzer (MA-6000P, Molarray Molecular Diagnostics) and amplification was terminated by heating the reaction to 80°C for 2 minutes. Assimilating Probes were used to estimate the real-time fluorescence signal every minute of the reaction.

LAMP sensitivity and specificity test

DNA isolated from Burkholderia pseudomallei was used to perform the TTSS1-orf2 LAMP sensitivity test. The concentration of the DNA from Burkholderia pseudomallei was measured and adjusted to 65 ng/μL using a spectrophotometer. The DNA was then diluted serially using sterile double-distilled water (ddH_2O) to 6.5 ng/μL, 650 pg/μL, 65 pg/μL, 6.5 pg/μL, and 650 fg/μL, and these DNA concentrations corresponded to bacterial concentrations of 1×10^7 cfu/mL, 1×10^6 cfu/mL, 1×10^5 cfu/mL, 1×10^4 cfu/mL, and 1×10^3 cfu/mL, respectively. The LAMP experiment was carried out in described above. Sterile ddH_2O was used as the negative control. The limits of detection of real-amp were determined using DNA obtained from Burkholderia pseudomallei. DNA was also isolated from 8 other bacterial strains to test the specificity of LAMP for Burkholderia pseudomallei TTSS1-orf2.

Results

LAMP primers designed in this study

Target selection for primer design was accomplished by using Primer Explorer (http://Primer

Table 1. Sequences of Primers for the Burkholderia pseudomallei orf2 gene in the real-amp assay

Primer 1	Sequence (5'-3')
F3	CTGCCTGACAATCCGCTA
B3	CGGTCAGTATCCGTAACAG
FIP (F1c + F2)	GACGCGTGTCGGCTCTTTATCGAGCGAATGACGGAT
BIP (B1c + B2)	GTCGAGCAATCGGCGGATCTTCATGGTCTCATTCCAGG
FLP	CGTAAATACGAAATCGACCAGG
BLP	GAATCTGGATCACCACCACTT
Primer 2	Sequence (5'-3')
F3	CAATCCGCTAGCAACAGAT
B3	CGGTCAGTATCCGTAACAG
FIP (F1c + F2)	TTGCTCGACAGTATAGAGACGCTGCATTCTTTCGCATACCC
BIP (B1c + B2)	CGGCGGATATCCGGAATCTGCTTCATGGTCTCATTCCAGG
FLP	CCGTAAATACGAAATCGACCAG
BLP	GATCACCACCACTTTCCGT
Primer 3	Sequence (5'-3')
F3	CAATCCGCTAGCAACAGAT
B3	CGGTCAGTATCCGTAACAG
FIP (F1c + F2)	GACAGTATAGAGACGCGTGTCGGAATGACGGATGCATTCTTTC
BIP (B1c + B2)	CGGCGGATATCCGGAATCTGCTTCATGGTCTCATTCCAGG
FLP	CGTAAATACGAAATCGACCAGG
BLP	GATCACCACCACTTTCCGT
Primer 4	Sequence (5'-3')
F3	CTGCCTGACAATCCGCTA
B3	CGGTCAGTATCCGTAACAG
FIP (F1c + F2)	CAGTATAGAGACGCGTGTCGGCGAATGACGGATGCATTCTT
BIP (B1c + B2)	TCGAGCAATCGGCGGATATCCTTCATGGTCTCATTCCAGG
FLP	CGTAAATACGAAATCGACCAGG
BLP	GAATCTGGATCACCACCACTT
Primer 5	Sequence (5'-3')
F3	CTGCCTGACAATCCGCTA
B3	CGGTCAGTATCCGTAACAG
FIP (F1c + F2)	TTGCTCGACAGTATAGAGACGCCGGATGCATTCTTTCGCA
BIP (B1c + B2)	CGGCGGATATCCGGAATCTGCTTCATGGTCTCATTCCAGG
FLP	CGTAAATACGAAATCGACCAGG
BLP	GATCACCACCACTTTCCGT
Primer 6	Sequence (5'-3')
F3	CTGCCTGACAATCCGCTA
B3	CGGTCAGTATCCGTAACAG
FIP (F1c + F2)	GACAGTATAGAGACGCGTGTCGACGGATGCATTCTTTCGC
BIP (B1c + B2)	CGGCGGATATCCGGAATCTGCTTCATGGTCTCATTCCAGG
FLP	CGTAAATACGAAATCGACCAGG
BLP	GATCACCACCACTTTCCGT

Explorer.jp/e/v4manual/Index.Html). The primer specificity was checked using the basic local alignment search tool (BLAST) against human DNA and other bacterial sequences in the non redundant GenBank database. Primers used in this study are listed in **Table 1**.

Burkholderia pseudomallei was amplified within 20 minutes with a loop backward primer. The fluorescence peak typically persisted for about 40 minutes (**Figures 1A-C** and **2A-C**). No amplification was seen with negative control. The primer dimer were not detected in Primer 5, so Primer 5 was chosen for the subsequent experiments (**Figure 2C**).

Sensitivity of the real-amp method

Using DNA isolated from Burkholderia pseudomallei, the reliability and sensitivity of LAMP for TTSS1-orf2 amplification was evaluated, and amplification was detected 10 minutes after the reaction began and peaked at approximately 20 minutes. In addition, no signal was found in the negative control (ddH_2O). This indicates that LAMP is dependable for TTSS1-orf2 amplification.

To determine the sensitivity of the Burkholderia pseudomallei TTSS1-orf2 LAMP assay, 10-fold serial dilutions of the DNA template (ranging from 6.5 ng/μL to 650 fg/μL) were used in LAMP reactions (**Figure 3**). Amplification was detected 18 minutes after the reaction began when the initial DNA template was at a concentration of 1 pg/μL.

Specificity of the real-amp method

TTSS1-orf2 primers were specific to Burkholderia pseudomallei because no fluorescent signals, which indicate LAMP products, were detected in reactions containing DNA template from 8 other bacterial species (**Figure 4**). **Figure 4** shows that Acinetobacter baumannii, Staphyloccocus aureus, Klebsiella pneumonia,

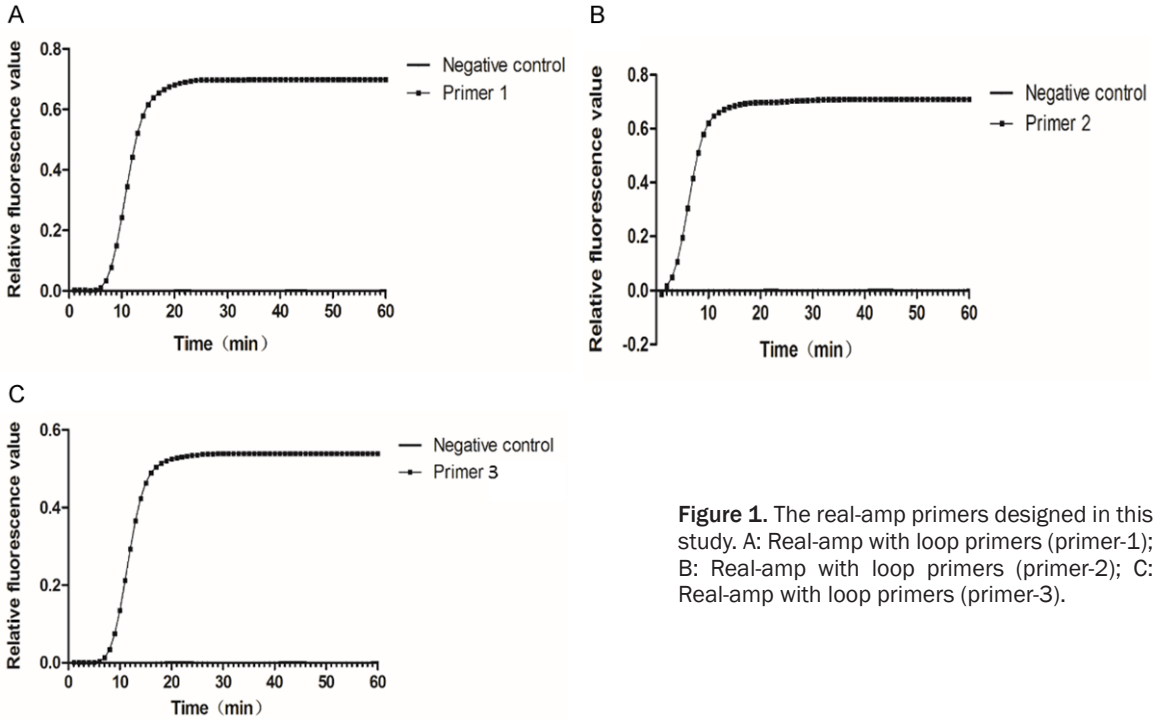

Figure 1. The real-amp primers designed in this study. A: Real-amp with loop primers (primer-1); B: Real-amp with loop primers (primer-2); C: Real-amp with loop primers (primer-3).

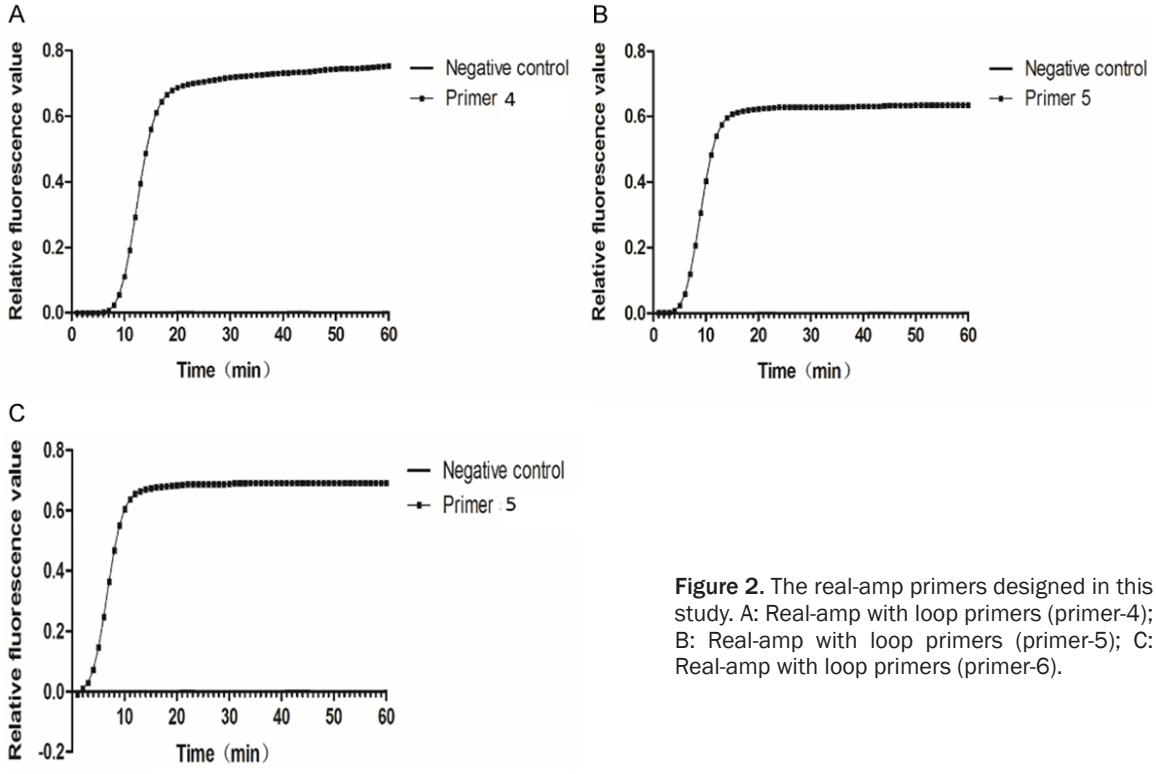

Figure 2. The real-amp primers designed in this study. A: Real-amp with loop primers (primer-4); B: Real-amp with loop primers (primer-5); C: Real-amp with loop primers (primer-6).

Streptococcus pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Streptococcus viridans and Haemophilus influenza were confirmed to be negative by real-amp assay.

Reproducibility of the real-amp method

The Burkholderia pseudomallei TTSS1-orf2 LAMP reactions were performed three indepen-

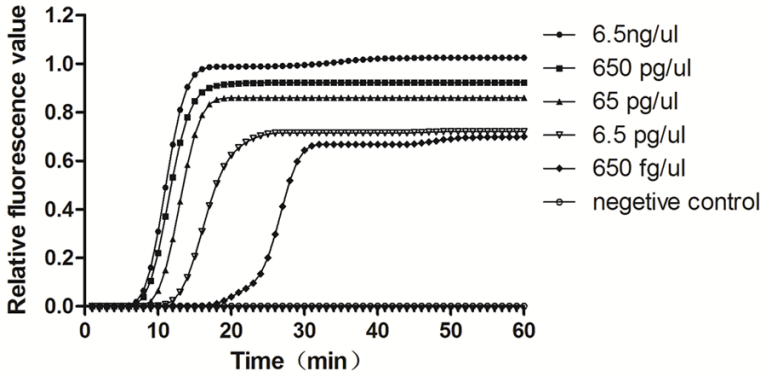

Figure 3. Sensitivity of the real-amp method.

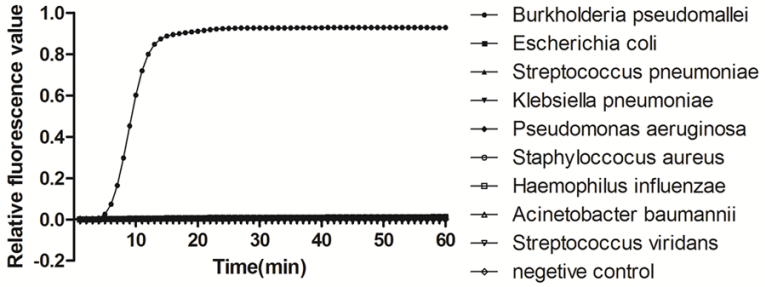

Figure 4. Specificity of the real-amp method.

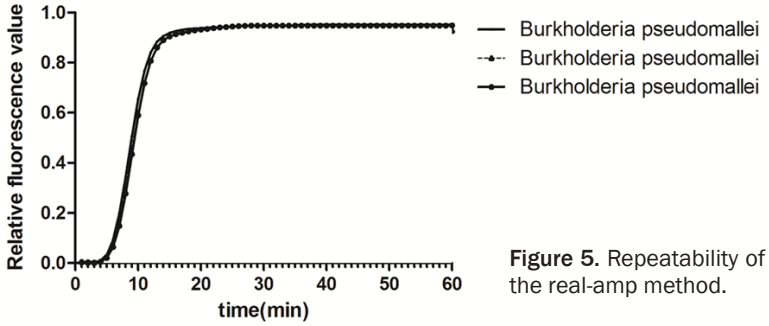

Figure 5. Repeatability of the real-amp method.

dent times and the results indicated that it is a stable reaction with a low CV of 2.55% (**Figure 5**).

Discussion

Laboratory identification of Burkholderia pseudomallei can be difficult, especially in Western countries where it is rarely seen. The large, wrinkled colonies look like environmental contaminants, so are often discarded as being without clinical significance [6]. Colony morphology is highly variable and a single strain may display multiple colony types, so inexperienced laboratory staff may mistakenly believe the growth is not pure. The organism grows more slowly than other bacteria that may be present in clinical specimens, and in specimens from nonsterile sites, is easily overgrown [17]. Even when the isolate is recognized to be significant, commonly used identification systems may misidentify the organism as Chromobacterium violaceum or other nonfermenting, Gram-negative bacilli such as Burkholderia cepacia or Pseudomonas aeruginosa. Molecular methods of diagnosis are possible, but not routinely available for clinical diagnosis [18].

Nucleic acid amplification is currently indispensable for precise clinical diagnoses, and as a result of technological advancements, it is now being utilized for rapid diagnoses of clinical pathogens [19]. The LAMP technique has been previously developed since its initial development in 2000 [20]. Among novel isothermal technologies, LAMP has the greatest potential for use in diagnostic medicine, has advantages over most PCR-based amplification tests, and has dramatically changed the application of molecular technologies, which were once considered difficult to implement in poorly resourced laboratories [21-23].

The aim of this study was to develop and evaluate a low-cost and effective method for detecting Burkholderia pseudomallei that could be utilized in developing tropical countries. Our results demonstrate that LAMP detects both reliably and sensitively the Burkholderia pseudomallei TTSS1-orf2 gene. To ensure specificity of the amplification reaction, unique primers are mandatory. The melting curves of our primers and amplifications with our primers demonstrated that our primers do not amplify non-specifi-

cally and do not form di-polymers, which ensures that our method was very specific.

The sequencing of the Burkholderia pseudomallei genome facilitated molecular diagnosis of melioidosis by molecular techniques, such as PCR [24]. All of the molecular techniques require selection of the appropriate target genes [25]. In this study, we chose the highly conserved TTSS1-orf2 gene in Burkholderia pseudomallei as the LAMP target gene. Although Burkholderia pseudomallei only has a single copy of TTSS1-orf2 in the genome, it is amplified efficiently and can be detected quickly. Consistently TTSS1-orf2 was detected 10 minutes after the reaction began and peaked at 20 minutes.

Traditional culture is generally regarded as the gold standard for identifying a Burkholderia pseudomallei infection [26], however, the sensitivity is low and the method is time-consuming [27, 28]. In this study, LAMP was highly sensitive when testing for the Burkholderia pseudomallei strain and LAMP could detect as little as 650 fg/μL of Burkholderia pseudomallei DNA, which is the equivalent of 1×10^3 cfu/mL. LAMP sensitivity is more likely to be affected by differences in DNA concentration and purity. Burkholderia pseudomallei DNA was isolated directly from pure bacterial colonies, whereas the clinical samples contained a lower bacterial load and the DNA isolated from the clinical samples was not as pure as the DNA isolated from Burkholderia pseudomallei.

The LAMP method requires much less time than traditional culture and is more cost-effective [29, 30]. In this study, the LAMP assay was shown to be completed in a short time and that the reaction products could be visualized by the naked eye. These results suggest that LAMP could be used as a simple, cost-effective, and reliable method for diagnosing melioidosis in developing countries. One limitation of this study is that the clinical sample size was quite small. A larger and more diverse sample size is necessary in order to accurately determine the sensitivity and reliability of this method.

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Disclosure of conflict of interest

None.

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