067 (D)

Loop-Mediated Isothermal Amplification (LAMP) for Rapid Detection of *Burkholderia pseudomallei*.

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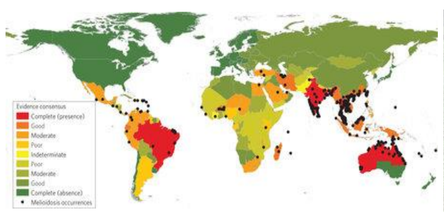
Abstract:

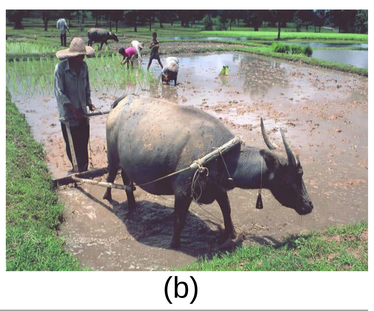
Burkholderia pseudomallei causes melioidosis, a deadly tropical disease that has been a major public health concern in Southeast Asia and northern Australia for decades. *B. pseudomallei* also has great biodefense implications due to its weaponization potential that can be dispersed effectively in air. Because there is no vaccine for melioidosis, successful patient management is solely based on early diagnosis and subsequent treatment with antibiotics. Although, *B. pseudomallei* can be found in soil throughout Southeast Asia, its disease is still not well-recognized. University of Florida and Hokkaido University have developed One Health Initiative and multi-disciplinary team approach on melioidosis investigations in Southeast Asia. One of the most urgent efforts is to develop a rapid test for ease identification of *B. pseudomallei* in clinical and environmental settings. A Loop-mediated Isothermal Amplification (LAMP) technique was developed to amplify *B. pseudomallei* – specific gene BPSS0135, a hypothetical protein gene, which is conserved across multiple genomes of *B. pseudomallei*. A panel of more than 500 DNA samples from culture confirmed clinical and environmental *B. pseudomallei* strains, and negative control DNA samples from a wide range of bacterial species were tested for sensitivity and specificity by LAMP. Our study has demonstrated that the amplification of this *B. pseudomallei*-specific gene was detected within 20 minutes with all *B. pseudomallei* DNA samples, while no amplification was detected with the negative control DNA. This rapid detection yields great potential for routinely use in melioidosis investigation.

Introduction:

Burkholderia pseudomallei is the causative agent of melioidosis, a severe tropical disease that is endemic in most tropical regions (Fig. 1a). *B. pseudomallei* is an aerobic, motile, Gram-negative bacillus. Its natural habitats are soil and water (Fig. 1b,c). The organisms can cause diseases in humans and animal via three different routes: percutaneous inoculation, ingestion, and inhalation. Melioidosis may have wide range of clinical manifestations and severity from an acute fulminant septic illness to a chronic infection (Fig. 1d).



(a) Limmathurotsakul *et al*. 2016



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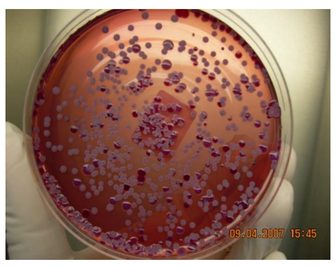

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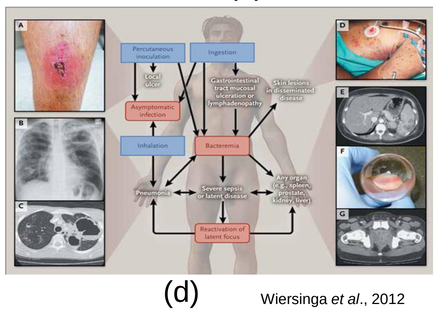


(d) Wiersinga *et al*., 2012

Fig. 1. Epidemiology, clinical, and environmental aspects of *B. pseudomallei*, the causative agents of melioidosis; (a) global distribution of melioidosis, (b) rice farming activities in Thailand, (c) *B. pseudomallei* isolated from soil in Northeast Thailand, and (d) various clinical manifestations and three different routes of infection. Melioidosis is mostly found in rice farmers. Most common forms of melioidosis are acute pneumonia and skin abscess. Natural habitats of *B. pseudomallei* are soil and surface water.

Significance:

- Melioidosis is a major international public health concern with high mortality rate, approximately 40% of treated patients in Thailand.
- *B. pseudomallei* is classified as a potential biothreat agent (Tier 1 Select Agent) in the United States.
- There is no effective vaccine. Successful patient management is solely based on early diagnosis and subsequent treatment with antibiotics.

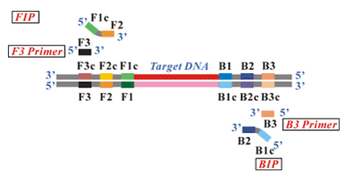
Objectives:

To develop a simple, reliable, and cost effective DNA-based rapid test that can be used to identify *B. pseudomalle*i in clinical and environmental laboratories.

Methodology:

LAMP (Loop-mediated isothermal amplification) for *B. pseudomallei*.

- LAMP used at least 4 primers specifically designed to recognize six distinct regions on the target gene.
- Amplification and detection of target gene can be completed in a single step, by incubating the mixture of sample, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (65ºC).
- It provides high amplification efficiency, with DNA being amplified 10^9-10^{10} times in 15 - 60 minutes. Because of its high specificity, the presence of the target gene sequence can easily be detected just by judging presence of amplified products.
- Gene BPSS0135, one of the conserved genes of *B. pseudomallei*, was chosen for LAMP assay development (Spring-Pearson et al. 2015).
- PrimerExplorer software was used to design LAMP primers.



BPSS0135 (420 bp)

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FIP: 5′- GCTCACGCACACGTCGGTCGTTCTTCGCGAAGGTGGCG -3′
BIP: 5′- GGCGGGCGATGGGCTACCTGCGTCGAACTCGAACACG -3′
F3:  5′- CGTTTCTCGGCCGGATGC-3′
B3:  5′- CGCTCGATGAGCCCGTTC-3′
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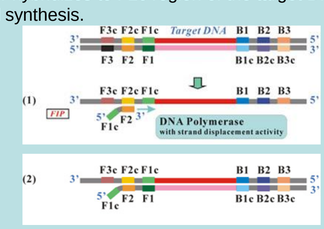
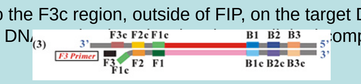
Stages in Loop-mediated Isothermal Amplification invented by Eiken Chemical Co., Ltd. (Japan)

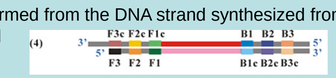
- F2 region of FIP primer hybridizes to F2c region of the target DNA and initiates complementary strand synthesis.



- F3 primer anneals to the F3c region, outside of FIP, on the target DNA and initiates strand displacement DNA synthesis. The newly synthesized DNA is complementary strand.

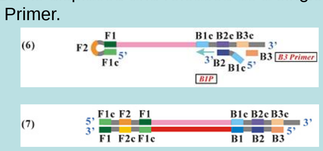


- A double strand is formed from the DNA strand synthesized from the F3 Primer and the template DNA strand

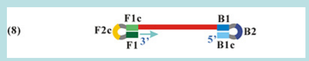


- The FIP-linked complementary strand is released as a single strand because of the displacement by the DNA strand synthesized from the F3 Primer. Then, this released single strand forms a stem-loop structure at the 5' end because of the complementary F1c and F1 regions.

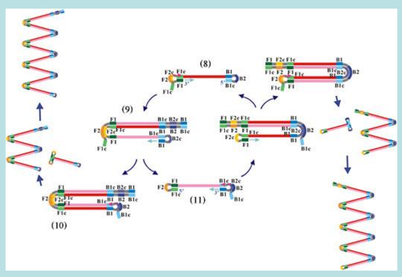
- This single strand DNA in Step (5) serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. The BIP anneals to the DNA strand produced in Step (5). Starting from the 3' end of the BIP, synthesis of complementary DNA takes place. Through this process, the DNA reverts from a loop structure into a linear structure. The B3 Primer anneals to the outside of the BIP and then, through the activity of the DNA polymerase and starting at the 3' end, the DNA synthesized from the BIP is displaced and released as a single strand before DNA synthesis from the B3 Primer.



- The BIP-linked complementary strand displaced in Step (6) forms a structure with stem-loops at each end, which looks like a dumbbell structure. This structure serves as the starting structure for the amplification cycle in the LAMP cycling.



- Cycling amplification.



- The final products obtained are a mixture of stem loop DNA with various stem lengths and various cauliflower like structures with multiple loops. The structures are formed by annealing between alternatively inverted repeats of the target sequence in the same strand.

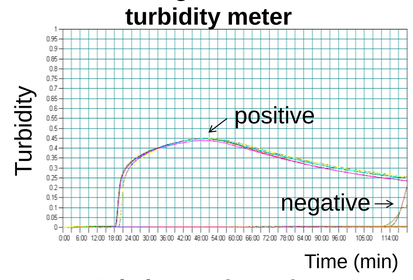
http://loopamp.eiken.co.jp/e/lamp/principle.html

Results:

Detection of the amplification

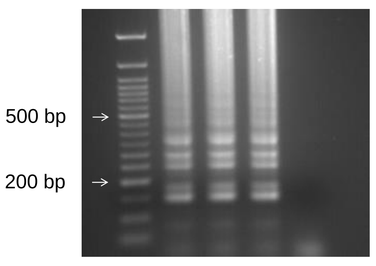
- Using a real-time turbidity meter
- Using Florescence detection reagent (Eiken Chemical, Japan)
- Gel electrophoresis

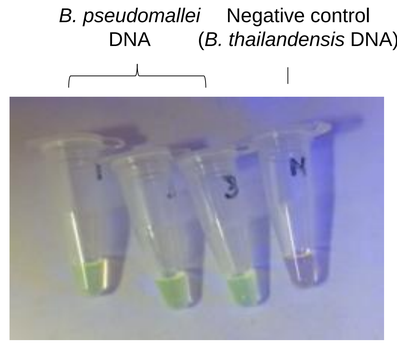
Using real-time turbidity meter



w/ Florescence detection

Gel electrophoresis





Sensitivity and Specificity:

- Limit of detection (LOD): 100 fg of DNA (10-15 bacilli) within 1 hr.
- Recommended DNA concentration: 10 ng of DNA within 20 min.
- Positive to *B. pseudomallei* DNA from clinical and environmental isolates (N=450).
- Negative to DNA from closely related bacterial species (N=20) e.g. *B. thailandensis*, *B. oklahomensis*, *B. cepacia* and *B. ubonensis.*
- Negative to DNA from other common bacterial pathogens in the tropics (N=20) e.g., *Leptospira spp*., *Acinetobacter spp*., *Mycobacterium spp*. and other common Gram-negative and Gram-positive bacteria (N=10).

Cost: Approx. $0.85 per LAMP reaction.

Conclusion:

- LAMP assay targeting BPSS0135 gene of *B. pseudomallei* has high specificity and sensitivity.
- Primers FIP and BIP are the key primers designed to generate a stem-loop structure (a dumbbell structure) on the DNA template strands mediating the LAMP cycling amplification.
- The annealing of primers F3 and B3 to flanking regions of FIP and BIP and the activity of *Bst* DNA polymerase initiated strand displacement DNA synthesis and releasing the FIP and BIP-linked complementary strands.
- The technique is cost effective and easy to use in a basic laboratory setting.

Future study

- Validation of LAMP assay in clinical and environmental specimens.
- Development of LAMP assays to detect drug resistance genes or their specific mutations in *B. pseudomallei.* This project was recently funded by CRDF Global and AMED under the U.S.-Japan Cooperative Medical Sciences Program Collaborative Awards, 2016.

References:

- Limmathurotsakul *et al*. 2016. Predicted global distribution of *Burkholderia pseudomallei* and burden of melioidosis**.** Nat Microbiol. 1:15008
- Wiersinga *et al*., 2012. Melioidosis. N Engl J Med 2012; 367:1035-1044
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