DEVELOPMENT OF RAPID DETECTION FOR *BURKHOLDERIA PSEUDOMALLEI* BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

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TITLE DEVELOPMENT OF RAPID DETECTION FOR BURKHOLDERIA PSEUDOMALLEI BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

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เรื่อง : การพัฒนาการตรวจหาเชื้อ *Burkholderia pseudomallei* อย่างรวดเร็วโดยเทคนิค loop-mediated isothermal amplification

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คำสำคัญ : *Burkholderia pseudomallei*, LAMP

เชื้อ *Burkholderia pseudomallei* เป็นเชื้อแกรมลบที่เป็นสาเหตุก่อโรคเมลิออยโดสิส ซึ่งเป็นโรคที่พบได้บ่อยในประเทศเขตร้อนทางเอเชียตะวันออกเฉียงใต้และประเทศออสเตรเลีย เมื่อมีการติดเชื้อจะก่อให้เกิดอาการทางคลินิกที่รุนแรง การตรวจทางห้องปฏิบัติการที่มีความรวดเร็วจึงมีความจำเป็นอย่างมากที่จะช่วยให้ได้รับการรักษาอย่างทันท่วงที โดยทั่วไปการตรวจหาเชื้อจะอาศัยการเพาะเลี้ยงหรืออาศัยปฏิกิริยาพีซีอาร์ โดยทั้งสองเทคนิคจำเป็นต้องใช้ประสบการณ์ในการตรวจและเครื่องพีซีอาร์มีบริการเฉพาะบางห้องปฏิบัติการเท่านั้น การศึกษานี้จึงมีวัตถุประสงค์ที่จะพัฒนาการตรวจหาเชื้อ *Burkholderia pseudomallei* โดยเทคนิค loop-mediated isothermal amplification (LAMP) ซึ่งเป็นเทคนิคทางชีวโมเลกุลที่เหมาะกับห้องปฏิบัติการที่มีข้อจำกัดด้านเครื่องมือและสามารถพัฒนาต่อไปเป็นการตรวจแบบ point-of-care ได้ ผลการศึกษาพบว่าเทคนิค LAMP ที่พัฒนาขึ้นมีขีดจำกัดการตรวจที่ความเข้มข้น 10 pg/ µl เมื่อทำการศึกษาในสิ่งส่งตรวจทางคลินิกพบว่ามีความไวร้อยละ 100 โดยไม่พบปฏิกิริยาข้ามระหว่างสายพันธ์หรือกับเชื้อแบคทีเรียชนิดอื่น เช่น *Salmonella spp. Shigella spp. Escherichia coli Klebsiella pneumonia Pseudomonas aeruginosa Burkholderia thailandesis* และ *Burkholderia mallei* (มีความจำเพาะร้อยละ 100) เมื่อพิจารณาถึงผลการศึกษาแสดงให้เห็นว่าเทคนิค LAMP ที่พัฒนาขึ้นสามารถพัฒนาไปสู่การตรวจ point-of-care ได้ต่อไป

ABSTRACT

TITLE : DEVELOPMENT OF RAPID DETECTION FOR *BURKHOLDERIA PSEUDOMALLEI* BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION
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KEYWORDS : *BURKHOLDERIA PSEUDOMALLEI*, LAMP

Burkholderia pseudomallei, a gram-negative bacteria, is the causative agent of melioidosis, a tropical infectious disease, which is endemic to Southeast Asia and Northeast Australia. Because of its clinical manifestation as septicemic and acute melioidosis which can progress up to the severe infection, a rapid laboratory diagnosis is needed in order to give the effective treatment to the patients confirmed as having infected by *B. pseudomallei* in timely manner. Culture and PCR assays are commonly used to detect *B. pseudomallei*, but both tests require experienced microbiologists and are only available in a few research laboratories worldwide. Despite the higher sensitivity than culture, PCR assays also require experienced personnel and positive controls, and are relatively costly for resource-limited settings. The aim of this study was to develop a rapid detection for *B. pseudomallei* by loop-mediated isothermal amplification (LAMP) which could be an affordable alternative molecular assay which can be used as point-of-care diagnostic test applicable in poor resource health system therefore achieving an effective treatment of patients in case. The LAMP developed in this study was sensitive and specific for the laboratory detection of *B. pseudomallei*. The lower limit of detection was 10 pg/µl and LAMP developed in this study was positive for 73 clinical samples collected from patients diagnosed with cultured-confirmed melioidosis (analytical sensitivity 100%), but it was negative for others bacteria used in this study including *Salmonella spp., Shigella spp., Escherishia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, B. thailandesis, and B. mallei.*

All 18 negative samples for melioidosis by bacterial culture were negative by LAMP (analytical specificity, 100%). The diagnostic accuracy of LAMP was 100%. Considering the specificity, sensitivity and diagnostic accuracy, the LAMP assay developed in this study can be used in health care system as a point-of-care laboratory assay for rapid detection of *B. pseudomallei*.

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LIST OF ABBREVIATIONS

CHARACTER	MEANING
ATP	Adenosine triphosphate
BIP	Backward inner primer
Bst	Bacillus stearothermophilus
B3	Backward outer primer
CLED	Cystine-lactose-electrolyte-deficient
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide phosphate
FIP	Forward inner Primer
FRET	Fluorescent resonance energy transfer
F3	Forward outer primer
HAD	Helicase-dependent amplification
IFAT	Indirect fluorescent antibody technique
IHAT	Indirect haemagglutination test
iNOS	Inducible nitric oxide synthase
LAMP	Loop-mediated isothermal amplification
LB	Loop backward
LF	Loop forward
LPS	Lypopolysaccharides
MDA	Multiple displacement amplification
MgCl2	Magnesium chloride
mM	Millimolar
MNGCs	Multinucleated giant cells
NASBA	Nucleic acid sequence-based amplification
oC	Celsius degree
PCR	Polymerase chain reaction
RCA	Rolling circle amplification
RNA	Ribonucleic acid

LIST OF ABBREVIATIONS (CONTINUED)

CHARACTER	MEANING
RPA	Recombinase polymerase amplification
TTS1	Type three secretion systems 1
T3SS	Type three secretion system
T6SS-1	Type 6 secretion system
UV	Ultraviolet
WGA	Whole genome amplification
µl	Microliter

CHAPTER 1
INTRODUCTION

1.1 Background and rational of the study

B. pseudomallei, a gram-negative bacillus, is the agent of melioidosis, a tropical infectious disease, which is endemic to Southeast Asia and Northeast Australia. It is commonly found in soil and water, and it has been isolated from a variety of clinical samples in endemic areas. Besides Southeast Asia and Northeast Australia, melioidosis has been reported in the South Pacific, Africa, India, the Middle East, Central America and South America (Bandeira TdJPG, et al., 2017).

B. pseudomallei is the causal agent of melioidosis (in Greek, "melis" means "distemper," "oid" means "resemblance," and "osis" means "condition"). Captain Alfred Whitmore and his assistant isolated this bacterium from morphine injectors at the Rangoon General Hospital in Burma in 1911–1912. This bacterium originates in muddy water as well as humid soil and is prevalent in many tropical countries. Melioidosis has not only become a serious veterinary problem but can infrequently affect humans (Samy RP, et al., 2017)

B. pseudomallei infections are almost exclusively acquired via contact with a contaminated environmental source. The potential use of *B. pseudomallei* as a biological weapon, combined with limited treatment options, difficulty in diagnosis and current lack of a vaccine which make this organism of substantial importance to public health in tropical regions worldwide (Aziz A, et al., 2017).

A major cause of mortality in Southeast Asia and Northern Australia, it is now recognized as a significant cause of disease in tropical regions worldwide. In-hospital mortality in Northeast Thailand is 40%. In acute forms, death can occur within 24–48 hours of the onset of symptoms using a model that mapped cases and environmental *B. pseudomallei*. The estimated global burden of melioidosis is 165,000 human melioidosis cases/pa, causing 89,000 deaths. Furthermore, the model predicts melioidosis under-reporting. Key risk factors are diabetes mellitus, alcohol consumption,

consumption, chronic renal and lung disease, and increasing age (Dunachie SJ, et al., 2017).

Melioidosis usually has an incubation period of 1 to 21 days (mean: 9 days) and causes a wide range of acute or chronic clinical manifestations, including pneumonia, abscesses in various organs, neurological manifestations, or severe septicemia. Since *B. pseudomallei* is intrinsically resistant to many antibiotics, it requires an immediate diagnosis followed by specific and prolonged antibiotic therapy (Kohler C, et al., 2016).

Culture and PCR assays are commonly used to detect *B. pseudomallei* in the environment, but both tests require experienced microbiologists and are only available in a few research laboratories worldwide. For the culture method, soil specimens are initially cultured in selective broth for 2 days, and then the upper layer of the broth is streaked on an agar plate and incubated for a further 7 days. Identifying *B. pseudomallei* on agar plates among other soil microbes is time-consuming and requires expertise and experience. For effective molecular detection, soil specimens are also first enriched in selective broth for 2 days prior to nucleic acid amplification. Despite the higher sensitivity than culture, PCR assays also require experienced personnel and positive controls, and are relatively costly for resource-limited settings. (Rongkard P, et al., 2016).

Lack of effective point-of-care diagnostic tests applicable in resource-poor endemic areas is a critical barrier to effective treatment, prevention and control of *B. pseudomallei* infection.

Laboratory diagnostic is important not only for the prescribing of effective drugs for appropriate patients in adequate doses for treatment the person in case but also for preventing the evolution of the antibiotic resistance of B. pseudomallei.

As *B. pseudomallei* can be identified by using the conventional microbiological testing, PCR, immunological and biochemical reactions and other tests, all these laboratory tests especially molecular methods still have some disadvantages, such as high cost equipment, expensive reagents, time-consuming complicated procedure assays and high skill laboratory personnel which restrict the application of PCR in point-of-care testing. For these reasons, we decided to develop a rapid detection test

for *B. pseudomallei* by loop-mediated isothermal amplification in order to overcome the difficulties above and to give our contribution for the molecular diagnosis of the *B. pseudomallei* in area with limited capacities and to contribute to the point-of-care testing in poor resource health system.

1.2 Hypothesis of the study

A developed detection test based on loop-mediated isothermal amplification (LAMP) could be useful for rapid detection of *B. pseudomallei*.

1.3 Objectives of study

1.3.1 To develop a LAMP assay for *B. pseudomallei* detection.

1.3.2 To compare analytical performance between a developed LAMP with culture method.

1.4 Scopes and limitations of the research

The scope of this study was to develop a rapid test for the detection of *B. pseudomallei* by loop-mediated isothermal amplification. This study was limited on the analysis of 74 clinical samples collected in Thailand which were used to determine the efficiency of using the loop-mediated isothermal amplification assay for detection of *B. pseudomallei.*

1.5 Research site

The samples have been collected from Sanpphasitthiprasong Hospital, Ubon Ratchathani Province in Thailand and the laboratory experiments on the collected clinical samples have been analyzed in UBU Research Laboratory, College of Medicine and Public Health, Ubon Ratchathani University, Thailand.

1.6 Anticipated Outcomes

We aimed to develop the loop-mediated isothermal amplification for the rapid detection of *B. pseudomallei.* This technique will contribute to the health system, this technique should be simple, rapid and with the cost-effectiveness test comparing to the PCR and can be used in endemic area with resource-limited as point-of-care testing and for field applications.

CHAPTER 2
LITERATURE REVIEWS

2.1 Scientific classification of *B. pseudomallei*

B. peudomallei specie is classified in genus of Burkholderia in the family of Burkholderiaceae which is in order of Burkholderiales belonging in class of beta proteobactera in Kingdom of Bacteria (Yabuuchi, et al.1992)

2.2 Morphology and structure *of B. pseudomallei*

B. pseudomallei. (also known as *Pseudomonas pseudomallei)* is a gram-negative, bipolar staining, aerobic, motile, rod-shaped bacterium (VirginiaTech, et al., 2006). *B. pseudomallei* measures 2–5 μm in length and 0.4–0.8 μm in diameter and is capable of self-propulsion using flagella. The bacteria can grow in a number of artificial nutrient environments, especially betaine and arginine containing ones. In vitro, optimal proliferation temperature is reported around $40^{O}C$ in neutral or slightly acidic environments (pH 6.8–7.0). The majority of strains are capable of fermentation of sugars without gas formation (most importantly, glucose and galactose; older cultures are reported to also metabolize maltose and starch). Bacteria produce both exo- and endotoxins (Haase A, et al., 1997).

2.3 *B. pseudomallei* genome

The complete genome of *B. pseudomallei* consists of two circular replicons of 4.07 Mb and 3.17 Mb each that have been designated chromosome 1 and chromosome 2 which encode 3,460 and 2,395 coding sequences (CDSs), respectively (Edwards TE, et al., 2010) as shown in Figure 2.1. Where appropriate, categories are shown as pairs of concentric circles representing both coding strands. Rings from outside to inside:

GIs represented by red segments; scale (in Mb); annotated CDSs colored according to predicted function; additional CDSs compared to the sequenced *B. mallei* strain ATCC 2944; the percentage of G + C content plot; (G–C)/(G + C) deviation plot (>0%, olive; <0%, purple). Color coding for CDSs: dark blue, pathogenicity/adaptation; black, energy metabolism; red, information transfer; dark green, surface-associated; cyan, degradation of large molecules; magenta, degradation of small molecules; yellow, central/intermediary metabolism; pale green, unknown; pale blue, regulators; orange, conserved hypothetical; brown, pseudo genes; pink, phage plus IS elements; gray, miscellaneous (Holden MT, et al., 2004).

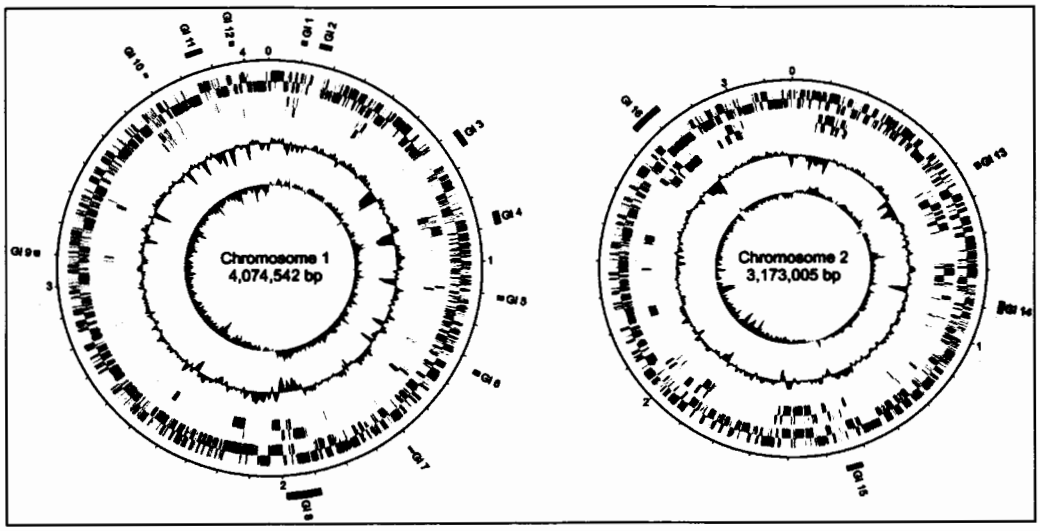


Figure 2.1 Schematic circular diagrams of the large and small chromosomes of the *B. pseudomallei* genome.

Source: Holden MT, et al. (2004)

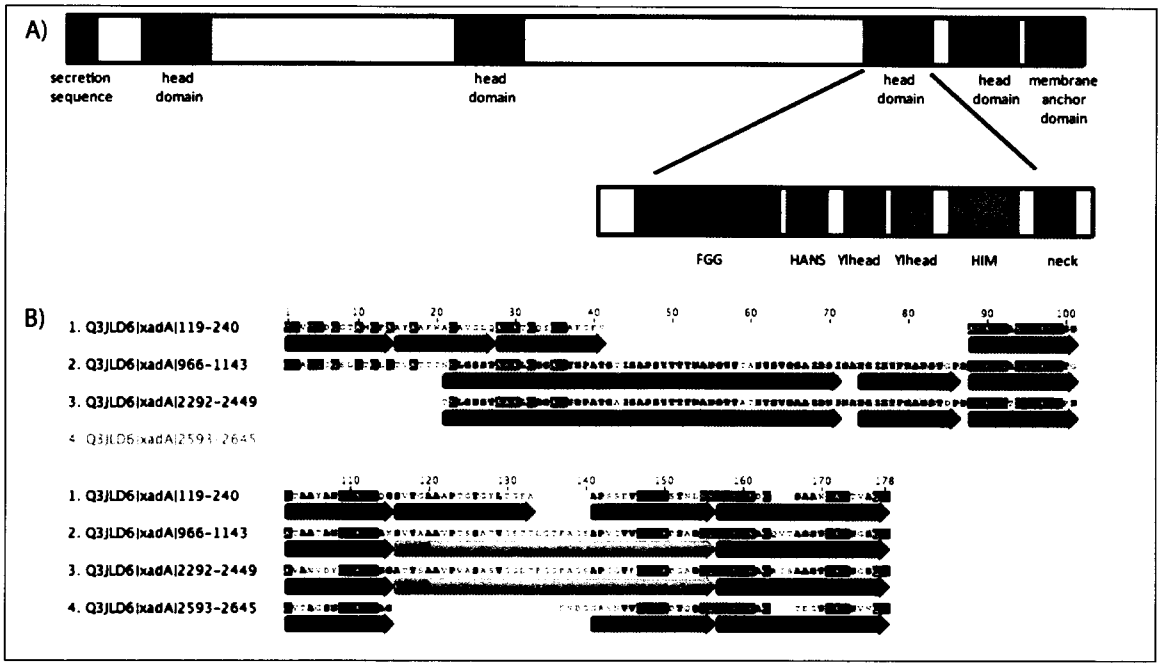


Figure 2.2 Primary structure and domain annotation of *BpaA* from *B. pseudomallei.*

Source: Edwards TE, et al. (2010).

(A) Domain architecture of the B. pseudomallei BpaA trimeric autotransporter adhesin (TAA). The BpaA TAA features an N-terminal secretion sequence, four head domains, and a C-terminal membrane anchored domain. The residues between head domains are identified as regions of low complexity and the region between the fourth head domain and the membrane anchored domain is likely to be a coiled-coil. The third head domain contains numerous sequence motifs identified by the domain annotation of Trimeric Autotransporter Adhesins (daTAA) server.

(B) Multiple sequence alignment of four head domains of B. pseudomallei BpaA. The four head domains were aligned according to their sequence and motifs identified by the daTAA are indicated

2.4 Geographical distribution

Melioidosis is caused by the *B. pseudomallei* and is mainly associated with tropical areas. Although considered endemic to Northern Australia and Southeast Asia, it has been increasingly recognized in other regions, such as Central America, South America, and the Caribbean (Gee JE, et al., 2017).

As of May 30, 2013, a total of 3,028 *B. pseudomallei* isolates were listed in the MLST database (www.mlst. net); these isolates are predominantly from Southeast Asia (1,036 isolates) and Australia (1,776). Some entries are from other Pacific areas (e.g., New Caledonia (9 isolates) and Hong Kong (39) and other parts of the world (e.g., Africa (8), Europe (15), and the Western Hemisphere (30) or of unknown origin (32), (Gee JE, et al., 2014).

Melioidosis is an emerging infectious disease that is being increasingly recognized in tropical regions around the world. While it is known to be endemic in at least 48 different countries in Southeast Asia, South Asia, the Middle East, Africa, Central America, and South America, current models predict that the disease is probably endemic in 34 additional countries where it is yet to be reported (Burtnick MN, et al., 2018).

2.5 Transmission and clinical features of melioidosis

Transmission of *B. pseudomallei* regularly occurs through inhalation, ingestion or direct contact with contaminated soil or water. Inoculation of the organism usually occurs through skin abrasions (S Cunha JP, 2010).

Human-to-human transmission is very rare. Humans are not the only susceptible host to *B. pseudomallei,* other animals include: sheep, goats, swine, horses, cats, dogs and cattle (Kozlowska J, et al., 2018). There have been few cases of zoonotic transmission; it is often transmitted through exposure to skin lesions by direct contact with infected animal either through meat or milk. Sexual transmission and vertical transmission have also been suggested in some cases but this is not common (Jilani M and, Alam S, 2017).

Melioidosis mainly affects susceptible persons who are directly in contact with contaminated wet soils. Immunosuppressed elderly persons (e.g., those suffering from diabetes mellitus and/or alcoholism) are at increased risk of developing infection. *B. pseudomallei* is also responsible for fibrosis and chronic lung diseases. The disease has variety manifestations ranging from localized abscess formation to disseminated abscesses, septicemia, shock, and possible death. The lungs are the most common organ affected by this disease; affected lungs lead to abscesses and septicemic spread. Many patients become acutely septicemic, as reported in Malaysia, Singapore,

Thailand, and Northern Australia. However, central nervous system involvement in melioidosis is rare. A number of septicemic patients have been also diagnosed with melioidosis. Several localized and septicemic melioidosis outbreaks also occurred after a Tsunami in 2004. A previous study showed that a Singapore Army soldier was also affected severely by cutaneous melioidosis (Bandeira TdJPG, et al., 2017).

2.6 Intracellular replication of *B. pseudomallei*

Intracellular living is also essential for localized spread from the site of infection, and eventual systemic dissemination. In brief, *B. pseudomallei* has the capability to invade cells either passively in the case of uptake by phagocytes such as resident tissue macrophages and recruited neutrophils; or actively in the case of epithelial cell infection. An advantageous trait of *B. pseudomallei* in this regard are its flagellae, which in wet soil or aquatic environments propel the bacilli against a concentration gradient toward nutrients that may otherwise be scarce; and in the mammalian host, enable penetration of protective mucous linings and surfactant to reach epithelial cells. Other factors such as the expression of Type IV pilli are well-recognized adhesion factors, and *B. pseudomallei* also possesses two further proteins, BoaA and BoaB that promote attachment to epithelial cells.

Host cell factors also play a role, such as protease-activated receptor 1, expressed by various cell types, that promotes cell ingress and is associated with *B. pseudomallei* growth and dissemination. *B. pseudomallei* is cytotoxic to dendritic cells (DC), monocytes and macrophages within hours of infection, with an absolute requirement for internalization, caspase-1 activation, and the Type Three Secretion System (T3SS). The *B. pseudomallei* flagella hook-associated protein also induces cytotoxicity. The capsule of *B. pseudomallei* is a well-studied virulence factor that confers protection against antibiotics and host immune effectors such as complement and antibody deposition.

The secreted protein effectors that are exported via the T3SS have diverse effects. For example, a cell-cycle inhibitor, CHBP (BPSS1385), is able to arrest host cell division, delay apoptosis and modify the cytoskeleton. BipC, located within the bsa locus, contributes to host cell adhesion and invasion, intracellular replication, cytotoxicity, phagosomal escape. Adjacent to bipC on the chromosome is bipB, which has

similarly been reported for its role in intracellular *B. pseudomallei* infection, including induction of MNGC and cell-to-cell spread (Novak RT, et al., 2006).

B. pseudomallei gains access to epithelial or phagocytic cells via flagella and adhesion-related virulence factors. *B. pseudomallei* then goes on to expresses an arsenal of virulence factors to combat the host response as shown in Figure 2.3. (I) Uptake and entry: either via phagocytic cells or epithelial cells. When entering non-phagocytic cells such as epithelial cells, *B. pseudomallei* can attach and enter host cells. Flagella, pilli, and other adhesins BoaA and BoaB are involved in this process. Passive uptake by tissue resident macrophages or neutrophils also occurs. (II) Survival and escape: *B. pseudomallei* once taken up by the host cell is enveloped in an endocytic vesicle or phagosome. Upon internalization, *B. pseudomallei* is held within a phagosome, where the T3SS is required for *B. pseudomallei* escape. Escape occurs before maturation and killing. Resistance to oxidative stress response from the host, and production of the serine protease ecotin are also crucial steps for phagosome survival and subsequent escape. (III) *B. pseudomallei* replication and dissemination to surrounding cells, and fusion of neighbouring cells into MNGC's. Once outside the phagosome, *B. pseudomallei* is able to replicate host actin is utilized for the polymerisation of elegantly structured Ena/VASP-like tails. These projections alongside the T6SS-1 result in successful dissemination to surrounding cells, resulting in the formation of multi-nucleated giant host cells (MNGC). Some of the host-combative responses are shown in red text, and the dashed-arrow indicates the connectivity between T3SS and T6SS. Actin tails are not structurally accurate (Novak RT, et al., 2006).

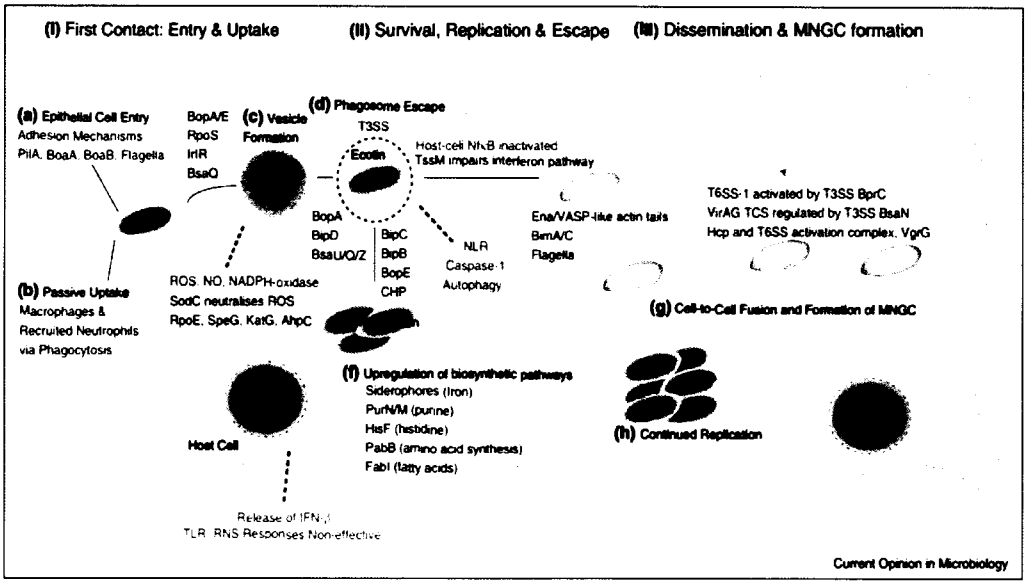


Figure 2.3 Intracellular life cycle of *B. pseudomallei*

Source: Willcocks SJ, et al. (2016).

2.7 Virulence and pathogenesis

B. pseudomallei is able to survive and replicate in macrophages, and is able to invade non-phagocytic cells, spread from cell-to-cell via actin-based motility and induce cell fusion (Jilani M and Alam, 2017).

After internalization in phagocytic cells, *B. pseudomallei* is able to escape from intracellular phagocytic and endocytic vacuoles, and followed by inducing actin polymerization to facilitate intracellular bacterial motility furthermore, this pathogen can inhibit host innate immune response by interfering the inducible nitric oxide synthase (iNOS) expression in mouse macrophage cell line (RAW 264.7), and can induce the multinucleated giant cells (MNGCs) formation for distributing bacterial infection to the adjacent cells before undergoing apoptotic cell death. Many virulence factors of *B. pseudomallei* have been characterized for the governing pathogenesis in host cells. RpoS sigma factor, one bacterial virulence factor has been studied the regulation in MNGCs formation and iNOS expression in both phagocytic and non-phagocytic cells (Joompa P, et al., 2017).

It is important to note that *B. pseudomallei* is a facultative intracellular pathogen and its ability to survive within host cells may be an important aspect of the disease chronicity (Bearss JJ, et al., 2017).

When *B. pseudomallei* cells come into contact with host cells, a plenty of host modifications occurs in response to bacterial infiltration and virulence factor expression eventually leading to a disease state. Within-host, *B. pseudomallei* can infect, invade and replicate inside the cytoplasm of many cell types. To accomplish this feat, *B. pseudomallei* attaches to the host cell via an unknown mechanism, causing actin rearrangement and inducing bacterial phagocytosis. Once attached, *B. pseudomallei* utilizes the Burkholderia secretion apparatus (*Bsa*), one of three genomically encoded type 3 secretion systems; T3SS) to secrete the effector BopE through the cytoplasmic membrane and this event is required for full virulence. BopE is a guanine nucleotide exchange factor for Rho family GTPases that undermines the surrounding cytoskeletal framework, inducing invasion of the host-cell by bacterial phagocytosis. The *Bsa* effector protein BopA then allows vesicular escape before phagosomal degradation of *B. pseudomallei* occurs. Once inside the cytoplasm the well characterized *B. pseudomallei*-intracellular motility protein A, (BimA), polymerizes host-cell actin. The polar actin "tail" allows intracellular movement and eventually leads to formation of membrane protrusions. The membrane protrusions allow *B. pseudomallei* to seek out uninfected cells and begin the cycle without transitioning into the extracellular milieu. During the infectious process, an infected cell may fuse with viable neighboring host cells and this is attributed to the main *B. pseudomallei* type 6 secretion system (T6SS-1). The formation of multi-nucleated giant cells (MNGCs) has been observed *in vitro* and *in vivo* and is a major hallmark of *B. pseudomallei* infection. The bacteria replicate concurrently with the production of several known and unknown virulence factors causing death of the host-cell. Due to high levels of genetic diversity and a flexible genome, not all virulence factors are universal; resulting in a variety of bacterial virulence factors expressed in vivo depending on the genetic background (Norris MH, et al., 2017).

The type three secretion systems 1 (TTS1) cluster is one of critical virulence factors that helps *B. pseudomallei* upon entry into the host cell for efficient escape from the endosome (Vander Broek CW and Stevens JM, 2017). *B. pseudomallei* is transmitted from its environmental reservoir to lung epithelial cells, where it initially attaches, possibly through bacterial components such as the capsule and type IV pili.Following invasion of epithelial cells, the T3SS-3 effectors assist in vacuolar

escape and intra-cellular motility due to BimA-mediated actin polymerization. The activation of TLR-2, TLR-4, and TLR-5 by bacterial LPS and flagella results in recruitment of innate immune cells, such as neutrophils, macrophages, and natural killer cells (Samy RP, et al., 2017).

The genome of *B. pseudomallei* consists of two circular chromosomes, the type three secretion systems 1 (TTS1) cluster, which is only present in *B. pseudomallei,* is residing on chromosome 2. The type three secretion systems 1 (TTS1) cluster is one of critical virulence factors that helps *B. pseudomallei* upon entry into the host cell for efficient escape from the endosome. The role of the T3SS is to deliver an array of effector proteins into the target cell to subvert host cell functions. The function of different effector proteins is extremely varied, ranging from blocking apoptosis, prevention of phagocytosis, cytotoxic activity and disruption of the actin cytoskeleton (Vander Broek CW, et al., 2017).

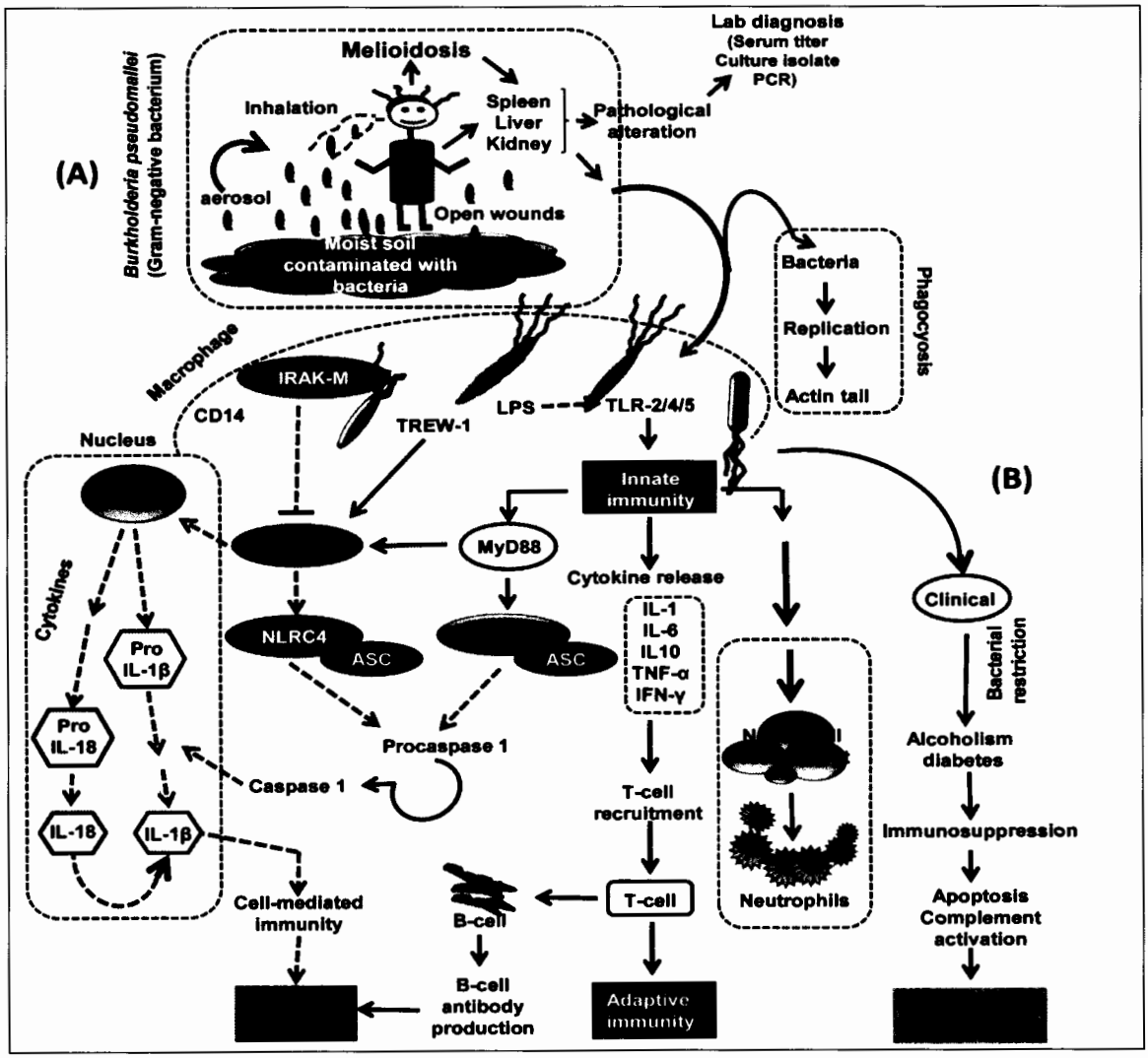


Figure 2.4 Schematic representation showing the virulence and pathogenesis of melioidosis infection.

Source: Samy RP, et al. (2017).

2.8 Laboratory diagnosis for *B. pseudomallei*

Melioidosis is usually diagnosed by isolation and identification of *B. pseudomallei* from the sputum, urine, tissues, blood samples, and wound exudates. The crucial need to make correct early diagnose in cases of melioidosis has always been problematic and is a dilemma for both clinicians as well as the laboratory pathologists. To date, this still relies on isolation and accurate identification of the pathogen from clinical specimens. Culture is considered as the gold standard even though a delay of 24 to 48 hours or more between the time of specimen plating and presumptive bacterial

identification. Several other serologic and molecular methods have been tested. Various techniques have been applied as complimentary diagnostic tools beside culture. A frequently employed test is serology, looking for specific antibodies.

Early serological tests developed by Nigg were the complement fixation test and the haemagglutination test (IHAT) using crude extracts of *B. pseudomallei* as antigen. A form of indirect fluorescent antibody technique (IFAT) was initiated by Ashdown to develop an IgG and IgM immunofluorescent test for melioidosis (Haque QM, 2010).

Though immunofluorescent antibody assay is rapid, highly sensitive and specific test for the diagnosis of infection, it requires a fluorescent microscope that is not always available in some laboratories. The PCR assay can be completed within I day, whereas cultures for *B. pseudomallei* typically require up to 5 days for identification of the bacteria. In addition to diagnostic applications, nucleic acid amplification procedures can also be modified as in real time PCR to allow for the quantitative measurement of bacterial load in order to monitor response to antibiotic therapy. In addition, the usefulness of PCR-mediated detection and identification of *B. pseudomallei* in clinical settings is highlighted. The researchers suggest that the LPS-PCR system can be applied not only for blood specimen, but to be extended to other clinical specimens such as sputum, urine, cerebrospinal fluid as a complement to culture (Haque QM, 2010).

2.8.1 Culture

Isolation of *B. pseudomallei* is readily achieved by using standard culture media such as blood agar, MacConkey agar, EMB, cystine-lactose-electrolyte-deficient (CLED) and routine blood culture broths, etc. Selective medium for *B. pseudomallei* is Ashdown's medium and selective broth. Cultures typically become positive in 24 to 48 hours for respiratory tract specimens to ensure reliable isolation from amongst the normal or contaminating flora. These media are easily prepared from common media constituents (Samy RP, et al., 2017).

Blood cultures have been confirmed negative just before death in the septicemic form of infection. This bacterium presents itself as a wrinkled colony on Ashdown's selective agar (ASA). The bacteria are gram-negative coccobacilli, with bipolar staining as observed in young cultures. Wrinkling of colonies is key to differentiation between *B. cepacia* (an opportunistic environmental pathogen) and *B.*

pseudomallei; thus, further confirmatory tests, such as polymerase chain reaction (PCR), may be needed (Bandeira TdJPG, et al., 2017).

Colonial morphology and simple biochemical tests will suggest the identity of the organism, which can then be confirmed by additional tests for 'non-fermenters'. Colonies are wrinkled, have a metallic appearance (as shown in Figure 2.5) and possess an earthy odour. On Gram's staining, the organism shows gram negative rod with characteristics "safety pin" appearance (bipolar staining as shown in Figure 2.6). Oxidase-positive bacillus resistant to gentamicin and colistin, but susceptible to amoxicillin/clavulanic acid. (Trinh T, et al., 2018).

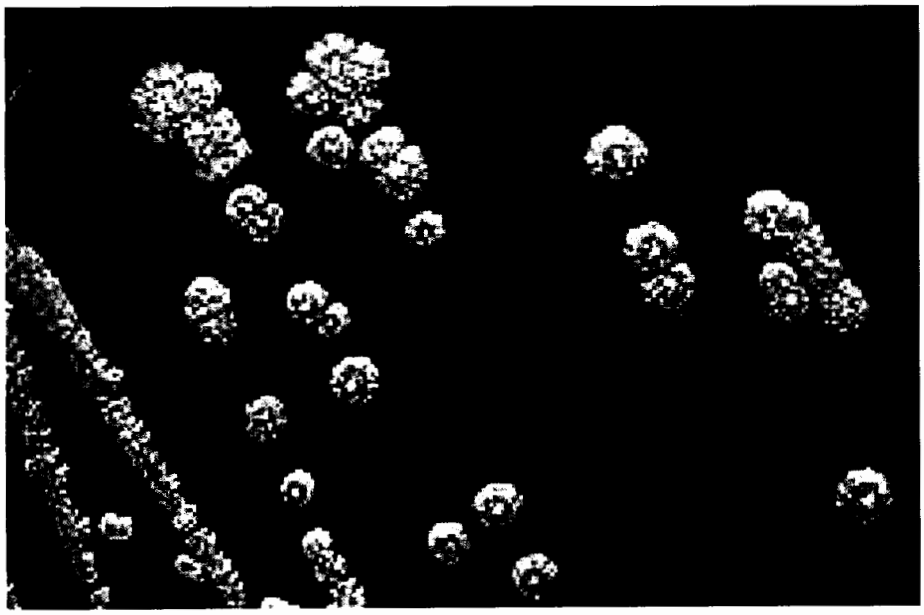


Figure 2.5 Colonies of *B. pseudomallei* on Ashdown's medium

Source: Trinh T, et al. (2018).

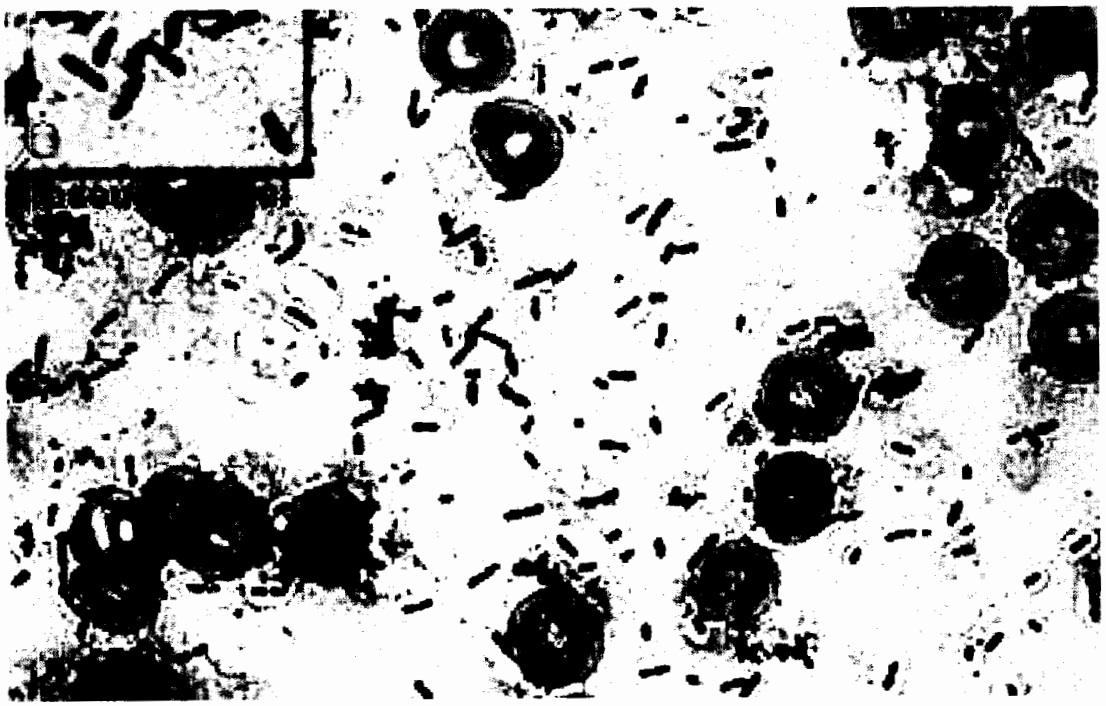


Figure 2.6 "Safety pin" appearance *B. pseudomallei* on Gram stain.

Source: Trinh T, et al. (2018).

2.8.2 Molecular tests

2.8.2.1 PCR

Polymerase Chain Reaction is a gene amplification or DNA amplification technique that have been widely applied in molecular biology laboratories including molecular biomedical research, diagnosis of infectious diseases and legal or environmental evidence proof. PCR is used in various ways such as real-time polymerase chain reaction (real-time PCR, qPCR), reverse transcription PCR (RT-PCR), nested PCR, and multiplex PCR. PCR is gold standard test based on gene amplification. This method can be used to detect DNA or RNA from various types of specimen such as live tissues, microbial cells, and cells in the cellular treatment. The major limitation of PCR is quality control. In addition, detection of specific DNA fragments by PCR is widely used in molecular diagnostics of various human and animal diseases, in forensics, food control, and analysis of environmental specimens (Garafutdinov RR, et al., 2017).

Real-time polymerase chain reaction (real-time PCR, qPCR) is also the quantitative gold standard test for gene amplification. The innovation of the real-time PCR technique played a crucial role in molecular medicine and clinical diagnostics. Examples are the quantitation of relative gene expression, detection of minimal residual disease (MRD), cancer diagnostics, pathogen detection, and quantitation of viral load in viral diseases (Overbergh L, et al., 2003).

PCR-based assay approach was developed to detect *B. pseudomallei* in clinical and soil samples isolates samples and it is used as confirmatory test. The real-time PCR assay targeting orf2 of *the B. pseudomallei* type III secretion system is a powerful tool for the rapid identification of *B. pseudomallei* and can potentially complement current confirmatory diagnostic procedures for melioidosis. (Novak RT, et al., 2006).

2.8.2.2 Isothermal DNA amplification techniques

The nucleic acid amplification is a key step in DNA detection assays. It contributes to improving the assay sensitivity by enabling the detection of a limited number of target molecules. The many different systems for nucleic acid analysis exploit the polymerase chain reaction (PCR) amplification method, which requires repeated cycles of three temperature steps during the amplification of the nucleic acid target sequence. In contrast, low temperature isothermal amplification methods have no need for thermal cycling thus requiring simplified microfluidic device features. Isothermal DNA amplification is an alternative to PCR-based technique. In isothermal techniques, amplification reactions are performed at a constant temperature and hence there is no need for expensive thermal cycling instrument. Isothermal DNA amplification techniques are simple, rapid, cost effective with equivalent specificity and sensitivity to PCR, and enabling point-of-care diagnostics without the need to high costing equipment. However, isothermal amplification approaches differed from each other in terms of operating temperature, duration of reaction, mechanism, strengths, and weaknesses.

A number of different isothermal amplification procedures have been introduced as nuclei acid amplification technique. These include nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), rolling circle amplification (RCA),

recombinase polymerase amplification (RPA) and multiple displacement amplification (MDA). Isothermal methods differ from PCR in that there is no need for temperature cycling or rapid heating and cooling mechanisms as in PCR systems. Many isothermal amplification systems exploit the strand-displacement activity of a DNA polymerase to cyclically amplify a target in less than an hour and different primers for several targets can be employed. In this study, only LAMP will be described in details as is one selected from isothermal amplification techniques to be used in developing the *B. pseudomallei* diagnostic tool and others will have only introduction.

1) Helicase-dependent amplification (HDA)

Helicase-dependent amplification (HDA) exploits the activity of a DNA helicase to separate complementary strands of double-stranded DNAs thus avoiding the temperature cycling to produce single-stranded templates for primer hybridization and subsequent primer extension by a DNA polymerase. It mimics the denaturation mechanism in living organisms where DNA is replicated by DNA polymerases with the aid of DNA helicase to separate complementary DNA strands (Zanoli LM and Spoto G, 2012). It mimics the replication fork and enables DNA synthesis to occur by using chemical energy. The helicase enzyme in the presence of ATP loads on to the dsDNA template and traverses along the target DNA, disrupting the hydrogen bonds linking the two strands. The formed single strand (ss) DNAs are then coated by single-stranded binding proteins (SSBs, step 1). Two sequence-specific primers hybridize to the 3'-end of each ssDNA template (step 2). DNA polymerases extend primers annealed to the target by producing dsDNA (step 3). The two newly synthesized dsDNA products act then as substrates for DNA helicases in the next round of the reaction (step 4), resulting in an exponential amplification of the selected target sequence (Zanoli LM and Spoto G, 2012) as shown in Figure 2.7.

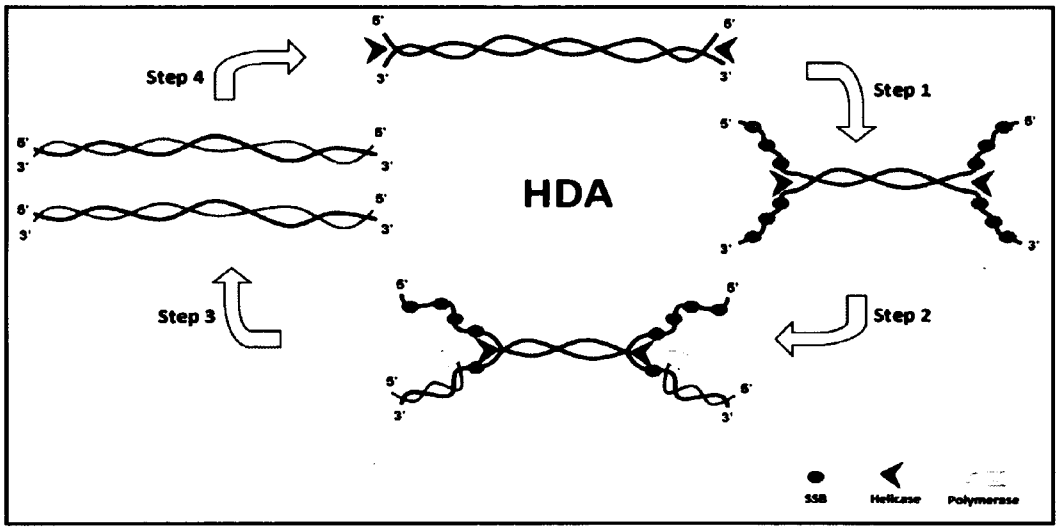


Figure 2.7 Schematic representation of helicase-dependent amplification process (HDA).

Source: Zanoli LM and Spoto G. (2012)

2) Rolling circle amplification (RCA)

The rolling circle amplification (RCA) method exploits the continuous amplification of a circular DNA template by a strand displacing DNA polymerase. The DNA polymerase displaces the synthesized strand and 'rolls' on with DNA synthesis. This method operates at a constant temperature and produces a long single-stranded DNA molecule with tandem repeats of the circular template. Both linear and exponential RCAs have been developed. In linear RCA, a small circle sequence is amplified by polymerase extension of a complementary primer, whereas in exponential RCA two primers are used: the second primer hybridizes with the single-stranded DNA product of the first primer and initiates hyper-branching in the DNA replication.

Efficient amplification by RCA requires small and circular single-stranded DNAs acting as the template. Unfortunately, a large number of relevant diagnostic DNA targets are composed of double stranded linear DNA molecules thus making it difficult the amplification process to be established. For this reason, oligonucleotides called padlock probes have been designed. Padlock probes are oligonucleotides with the two lateral sequences complementary to two target sequences that are connected by a linker sequence. When the padlock probe hybridizes

the target sequences it circularizes. Upon recognition of the target, the padlock segments are sealed through the action of a DNA ligase alone or in combination with a DNA polymerase. As the circularizing step of a padlock probe is strictly target-dependent, high enough specificity is ensured to allow SNP analysis.

Sample pre-treatments are required before the padlock probe circularization in order to allow the transformation of duplex genomic DNA into accessible single-stranded DNA targets. For this reason, genomic DNA is first digested with restriction enzymes that produce small sized dsDNA, then subjected to degradation by 5'-3' exonucleolysis. Even mRNA can be amplified through the RCA approach but, in this case, an mRNA pre-treatment is performed which consists of cDNA synthesis by a reverse-transcription followed by mRNA degradation by RNase H digestion. In both cases, RCA of padlock probes produces a large DNA product that collapses into a submicrometer-sized concatamer that can be tagged and analyzed for single molecule detection. The creation of one rolling circle product (RCP) for each recognized target makes optical detection and quantification possible by using fluorescence optical microscopy (Zanoli LM and Spoto G, 2012).

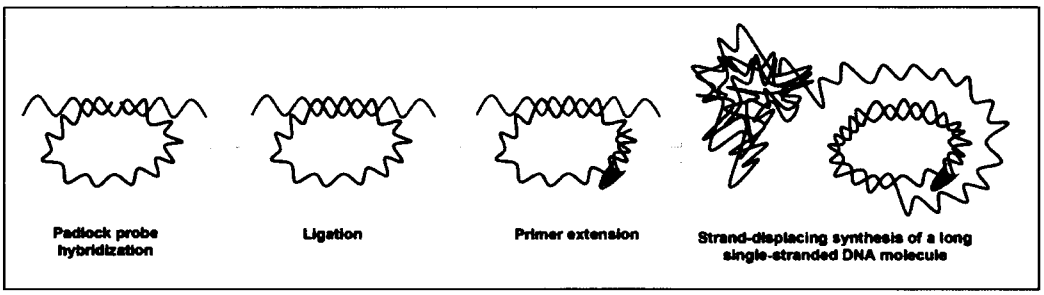


Figure 2.8 principal mechanisms for rolling circle amplification (RCA).

3) Multiple displacement amplification (MDA)

MDA is an isothermal method for whole genome amplification (WGA) capable of producing a large number of amplification products from a few DNA molecules. MDA exploits both random exonuclease-resistant primers as well as the strand displacement activity of φ29 DNA polymerase to produce DNA strands 70 kb in length. With no need for thermal cycling, random primers and φ29 DNA polymerase are already used for circular DNA amplification in RCA. In MDA, the

above-mentioned components are employed to amplify linear genomic DNA. The use of a high-fidelity proofreading DNA polymerase ensures a significantly lower error rate during the amplification reaction (3×10^{-6} mutations/nucleotide) in comparison with Taq DNA polymerase used for PCR amplification ($\sim2 \times 10^{-4}$ mutations/nucleotide). The high-fidelity amplification by MDA preserves DNA sequence information, assuring accurate genotyping in downstream applications regardless of the type of clinical sample used including buccal swabs, whole blood, finger stick blood and Guthrie cards.

MDA does not require the temperature-modulated denaturation of the genomic DNA to facilitate the annealing of random primers. In fact, the random priming on the double-stranded DNA target is efficient enough to obtain an initial priming step that is followed by the strand-displacement process that produces the amplification. It has been also demonstrated that the omission of the denaturation step results in a reduced template degradation and in an improved specificity of the amplification. MDA has been directly used on lysed cells thus avoids the DNA template loss and damage and the risk of contamination that can occur in DNA purification processes. The possibility to directly apply MDA to genomic material obtained from single cells helps in eliminating the need to develop culture methods by also allowing the direct analysis of genomic DNA from nonculturable bacteria. MDA has been shown to produce several-billion-fold amplification of genomic DNA from single, flow-sorted bacterial cells.

The isothermal amplification of DNA in minimally buffered conditions allows to perform and monitor nucleic acid amplification with minimal technological and operative requirements. The phi29 can operate multiple displacement amplification in minimally buffered conditions producing, as a readout, pH shifts attaining subunits of pH (Tenaglia E, et al., 2018).

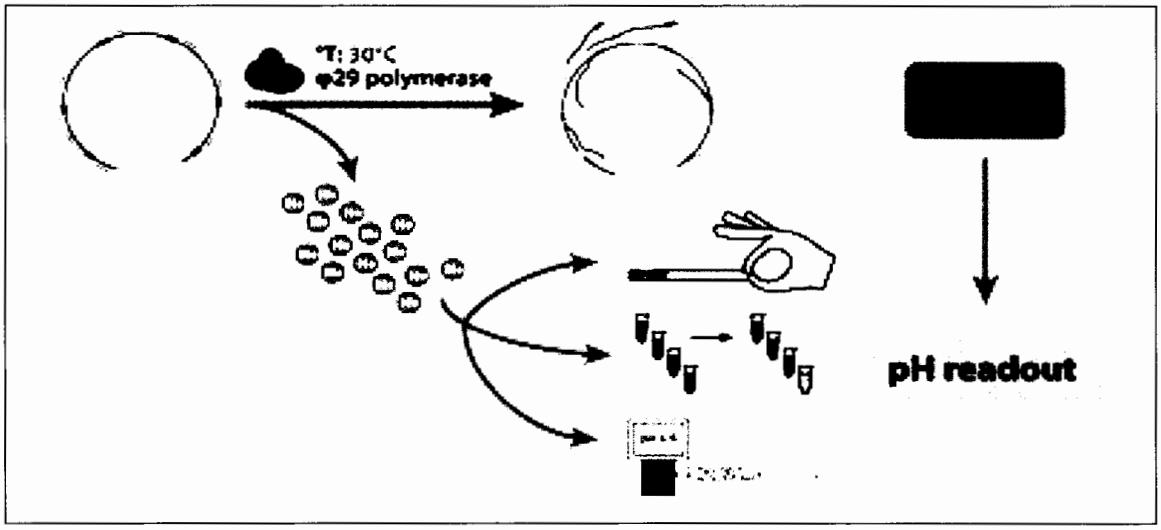


Figure 2.9 Multiple Displacement Amplification mechanism (MDA).

Source: Tenaglia E, et al. (2018)

4) Recombinase polymerase amplification (RPA)

RPA is a low temperature (about 30-37°C) isothermal amplification method that couples the isothermal recombinase-driven primer targeting of the template material with the strand-displacement DNA synthesis. In this method, combination with a novel probe-based detection approach amplifies DNA sequences by using a recombinase, DNA polymerase and DNA-binding proteins.

RPA uses nucleoprotein complexes constituted by oligonucleotide primers and recombinase proteins to facilitate the primer binding to the template DNA. In particular, recombinase-primer complexes scan the double-stranded DNA by promoting the primer binding at the target sequence of dsDNA and the displacement of the non-template strand. The displaced strand is stabilized by ssDNA binding proteins while the recombinase disassembly leaves 3'-end of the primer accessible to DNA polymerase. An exponential amplification of the target sequence is obtained after the cyclic replication of the process (Giuffrida MCand Spoto G, 2017).

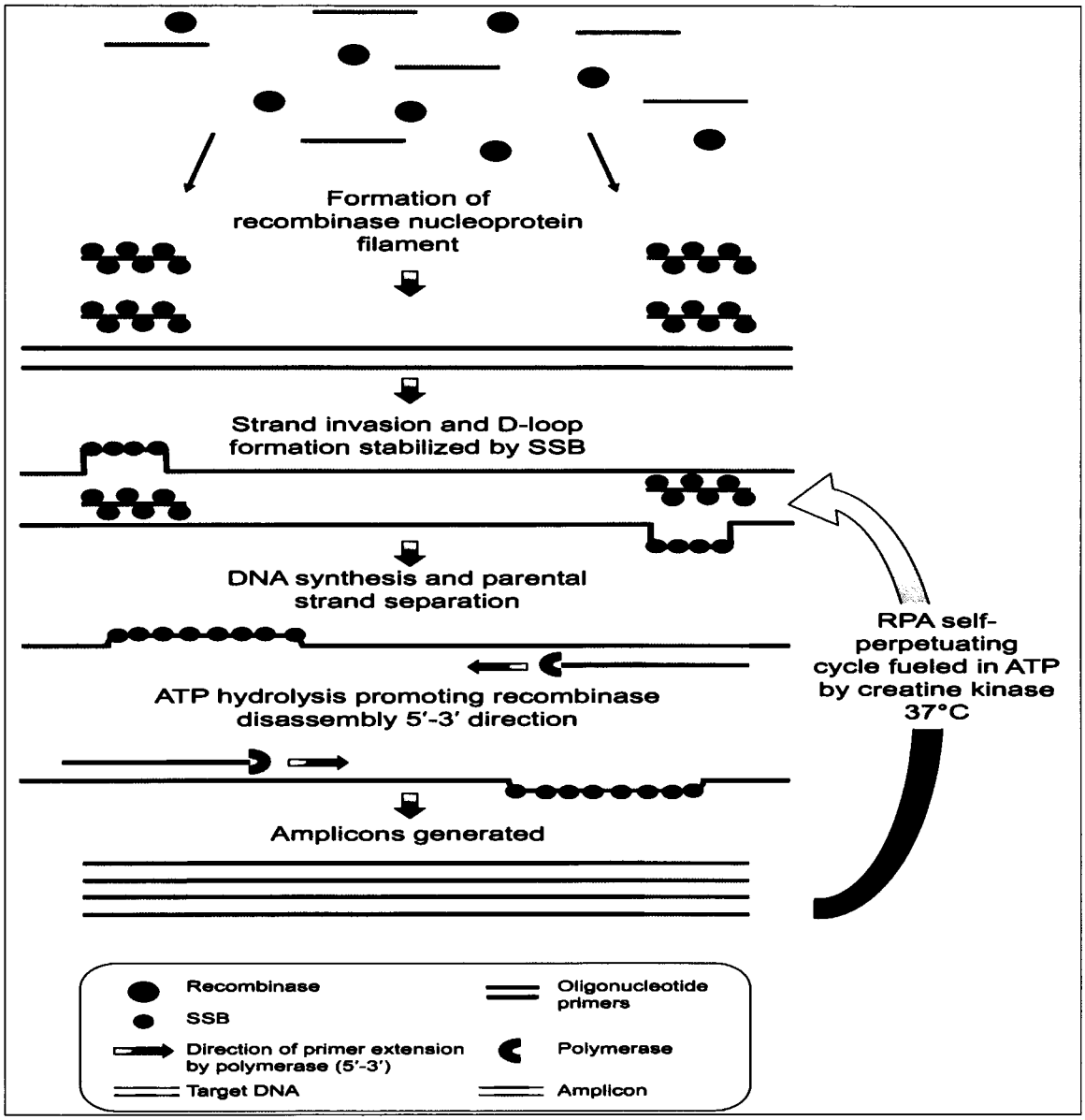


Figure 2.10 Schematic representation of RPA process.

Source: Giuffrida MCand Spoto G. (2017).

5) Nucleic acid sequence-based amplification (NASBA)

NASBA is an isothermal, transcription-based amplification method specifically designed for the amplification of single-stranded RNA or DNA sequence. It is performed at 41 °C. With the integration of the reverse transcription activity into the amplification process, the method is especially suited for RNAs such as mRNA, rRNA, tmRNA or genomic RNA. However, NASBA cannot amplify double-stranded DNAs because it is not subjected to a denaturation step.

NASBA utilizes two RNA target-specific primers and three enzymes (*i.e.*, avian myeloblastosis virus reverse transcriptase, T7 DNA-dependent RNA polymerase (DdRp) and RNase H). The standard NASBA protocol for RNA amplification requires a 65 °C RNA incubation step to denature the target prior to the addition of enzymes.

In the initiation phase, a specific forward primer (P1), that possesses a 5' sequence corresponding to the promoter of the T7 DdRp, hybridizes to any target RNA present in the sample and is extended by the reverse transcriptase. Subsequently, the RNA portion of the resulting RNA: DNA heteroduplex is degraded by RNase H, while a specific reverse primer (P2) hybridizes to the complementary sequence and is extended by the reverse transcriptase, leading to the formation of a dsDNA with the target sequence and a T7 promoter. Then, the T7 DdRp produces many RNA molecules that are complementary to the original target RNA. In the amplification phase, each newly synthesized RNA can be copied, resulting in an exponential amplification of RNA complimentary to the target (Zanoli LM and Spoto G, 2012). Single-stranded RNA products are detected by using hybridization-based detection protocols with sequence-specific probes. All processes are shown in Figure 2.11.

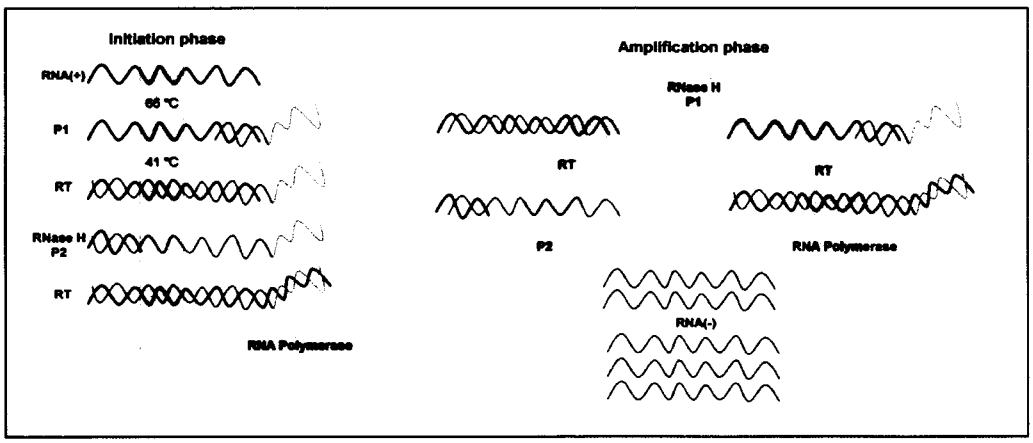


Figure 2.11 Schematic representation of nucleic acid sequence-based amplification (NASBA).

Source: Zanoli LM and Spoto G. (2012).

6) Loop-mediated isothermal amplification

Since the inception and development of (PCR) in the 1980s, this gene amplification method has been developed for widespread clinical use, particularly in genetic testing. This is especially true in the diagnosis of infectious diseases, and hereditary diseases. The steps of genetic testing include nucleic acid extraction from the specimens, gene amplification, and detection. These steps require considerable skill and expensive equipment and facilities, making convenient testing at any given location difficult. To overcome these limitations, a new gene amplification method, the loop-mediated isothermal amplification (LAMP) reaction, which combines rapidity, simplicity, and high specificity have been developed. Simple tests also have been developed based entirely on this technique (Notomi T, et al., 2015).

Loop-mediated isothermal amplification is a simple, rapid, specific and cost-effective nucleic acid amplification method solely developed by Eiken Chemical Co., Ltd. It is characterized by the use of 4 different primers specifically designed to recognize 6 distinct regions on the target gene and the reaction process proceeds at a constant temperature using strand displacement reaction. Amplification and detection of gene can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (about 65°C). 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 amplified product can indicate the presence of target gene (http://loopamp.eiken.co.jp/e/index.html)

6.1) Characteristics of LAMP

During LAMP there is no need for a step to denature double stranded into a single stranded form, the whole amplification reaction takes place continuously under isothermal conditions, the amplification efficiency is extremely high, by designing 4 primers to recognize 6 distinct regions, the LAMP method is able to specifically amplify the target gene.

The total cost can be reduced, as LAMP dose not require special reagents or sophisticated equipment, the amplified products have a structure consisting of alternately inverted repeats of the target sequence on the same strand,

amplification can be done with RNA templates following the same procedure as with DNA templates, simply through the addition of reverse transcriptase (Notomi T, et al., 2015).

6.2) Design of primers

LAMP requires 4 types of primers along with 2 additional loop primers to identify six distinct regions. Design 4 types of primers with 2 additional loop primers (described in detail below) based on the following 6 distinct regions of the target gene: the F3c, F2c and F1c regions at the 3' side and the B1, B2 and B3 regions at the 5' side as shown in Figure 2.12.

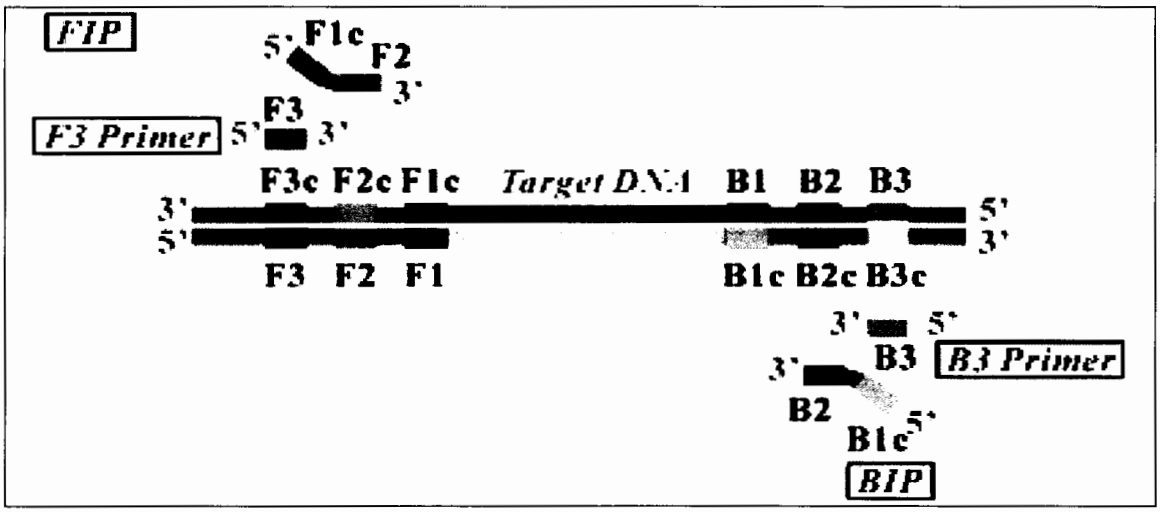


Figure 2.12 six types of primers based on the 6 distinct regions of the target gene

Source: Parida M, and et al. (2008)

FIP : Forward Inner Primer (FIP) consists of the F2 region (at the 3' end) that is complementary to the F2c region, and the same sequence as the F1c region at the 5' end.

F3 Primer : Forward Outer Primer consists of the F3 region that is complementary to the F3c region.

BIP : Backward Inner Primer (BIP) consists of the B2 region (at the 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end.

B3 Primer : Backward Outer Primer consists of the B3 region that is complementary to the B3c region

6.3) Basic principle and mechanism of the LAMP reaction

Loop-mediated isothermal amplification (LAMP) has been considered as an innovative gene amplification technology and is used as an alternative molecular technique to PCR-based methodologies in different areas of application including clinical laboratories, food products and environmental testing. In our days as a new developed gene amplification method, LAMP is used for diagnostic and identification of pathogens from various diseases because of its considerable advantages including cost-effective nucleic acids amplification method, simplicity, rapidity, high sensitivity and high specificity. Several tests have been developed based on this method, and simplicity is maintained throughout all steps, from extraction of nucleic acids to the detection of amplicons. The LAMP reaction has a wide range of possible applications, including point-of-care testing, field operations testing and genetic testing in resource-poor settings such as in developing countries.

The principle of LAMP is based on amplifying the gene by using 4 primers that can detect 6 positions of the target genes. Amplification process is performed under constant temperature (55-65 °C) with strand displacement reaction of DNA polymerase. The mechanism of LAMP consists of 2 steps; starting structure producing step and cycle amplification step. Firstly, production of starting structure requires all four primers (F3, B3, FIP, and BIP) to generate stem-loop. As shown in Figure 2.11, one of the inner primer (FIP) works first and initiating the replicative DNA synthesis by Bst DNA polymerase. Next the outer primer, F3, take its place, displacing the new synthesized strand and releasing the target DNA. With similar process of BIP and B3, this results in the single-strand stem-loop (dumbbell-like-structure with loops at both ends). This structure is starting material for next cycle amplification step. In cycle amplification step, the starting structure is amplified by self-primed DNA synthesis. FIP hybridizes and primes stand displacement DNA so that the complement of stem-loop is produced and elongated through recycling step reaction as shown in Figure 2.12. It provides high amplification efficiency, with DNA being amplified 10^9-10^{10} times in 15-60 minutes.

LAMP product can be monitored in one step without gel electrophoresis. The LAMP reaction can be visually observed by turbidity of

magnesium pyrophosphate. However, the LAMP still have some disadvantages, particularly, the sophistication of primer design.

When the target gene (DNA template as example) and the reagents are incubated at a constant temperature between 60-65°C, the following reaction steps proceed:

6.3.1) STEP 1

As double stranded DNA is in the condition of dynamic equilibrium at the temperature around 65°C, one of the LAMP primers can anneal to the complimentary sequence of double stranded target DNA, then initiates DNA synthesis using the DNA polymerase with strand displacement activity, displacing and releasing a single stranded DNA as shown in Figure 2.13. With the LAMP method, unlike with PCR, there is no need for heat denaturation of the double stranded DNA into a single strand. The following amplification mechanism explains from when the FIP anneals to such released single stranded template DNA.

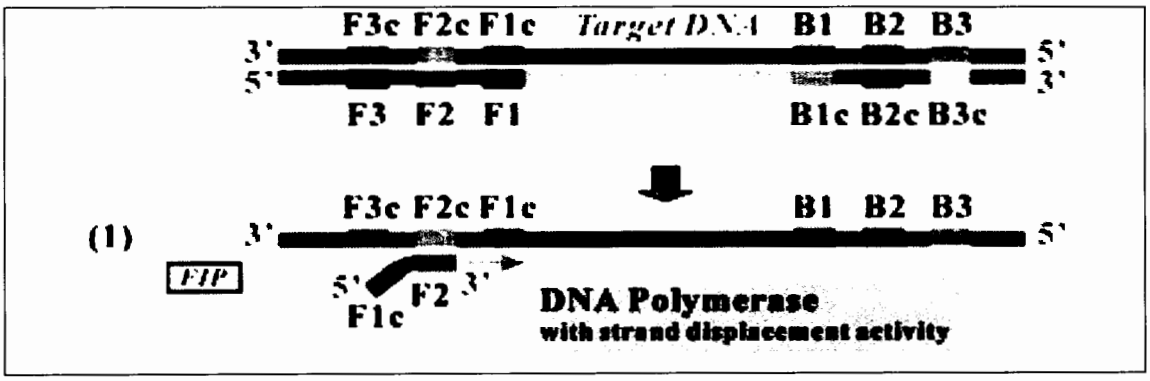


Figure 2. 13 Mechanism of LAMP reaction step1.

Source: Parida M, and et al. (2008)

6.3.2) STEP 2

Through the activity of DNA polymerase with strand displacement activity, a DNA strand complementary to the template DNA is synthesized, starting from the 3' end of the F2 region of the FIP as shown on Figure 2.14.

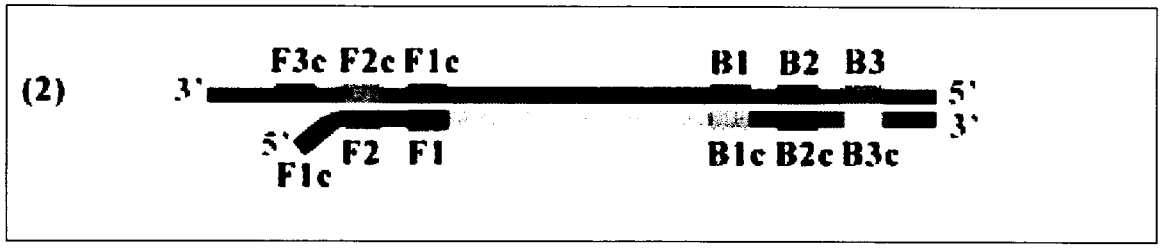


Figure 2.14 Mechanism of LAMP reaction step2.

Source: Parida M, and et al. (2008)

6.3.3) STEP 3

The F3 Primer anneals to the F3c region, outside of FIP, on the target DNA and initiates strand displacement DNA synthesis, releasing the FIP-linked complementary strand as shown in Figure 2.15.

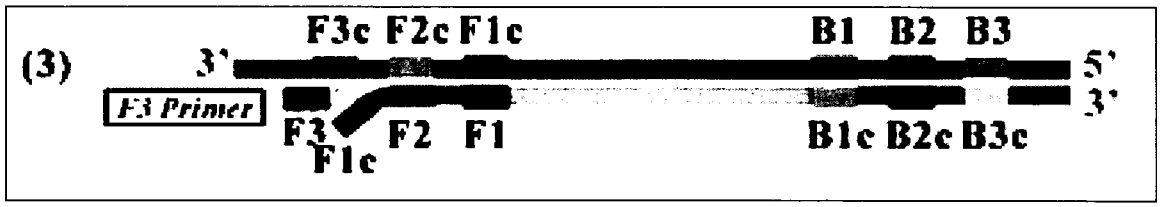


Figure 2.15 Mechanism of LAMP reaction step3.

Source: Parida M, and et al. (2008)

6.3.4) STEP 4

A double strand is formed from the DNA strand synthesized from the F3 Primer and the template DNA strand as shown in Figure 2.16.

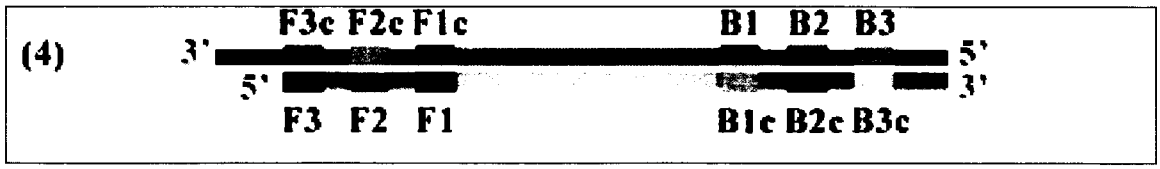


Figure 2.16 Mechanism of LAMP reaction step4.

Source: Parida M, and et al. (2008)

6.3.5) STEP 5

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 as shown in Figure 2.17

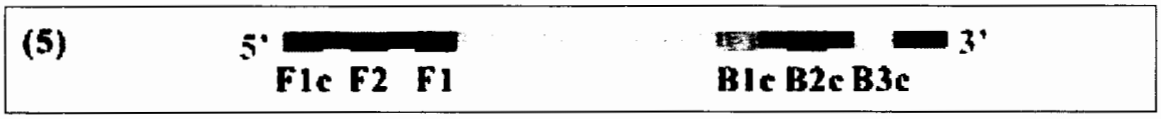


Figure 2.17 Mechanism of LAMP reaction step5.

Source: Parida M, and et al. (2008)

6.3.6) STEP 6

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 as shown in Figure 2.18.

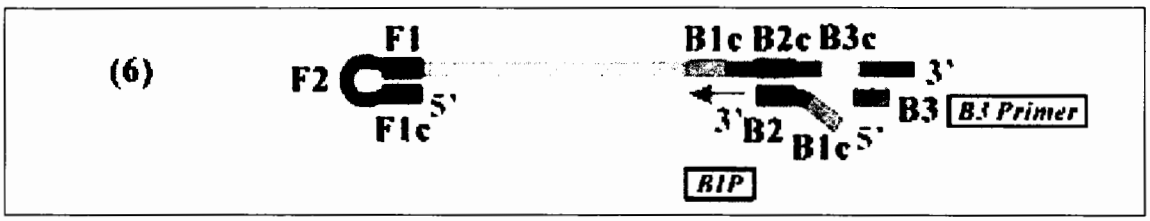


Figure 2.18 Mechanism of LAMP reaction step6.

Source: Parida M, and et al. (2008)

6.3.7) STEP 7

Double stranded DNA is produced through the processes described in Step (6) and seen in Figure 2.19.

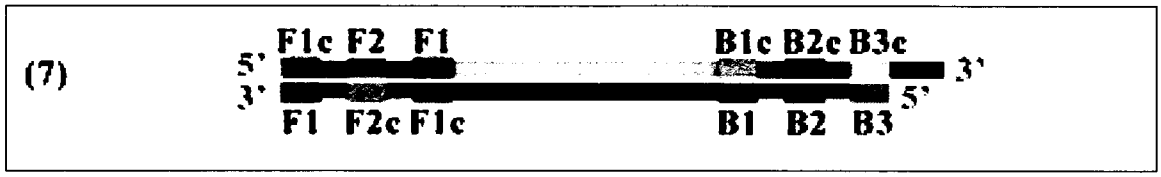


Figure 2.19 Mechanism of LAMP reaction step7.
Source: Parida M, and et al. (2008)

6.3.8) STEP 8

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 method (LAMP cycling). The above process can be understood as producing the starting structure for LAMP cycling as shown on Figure 2.20.

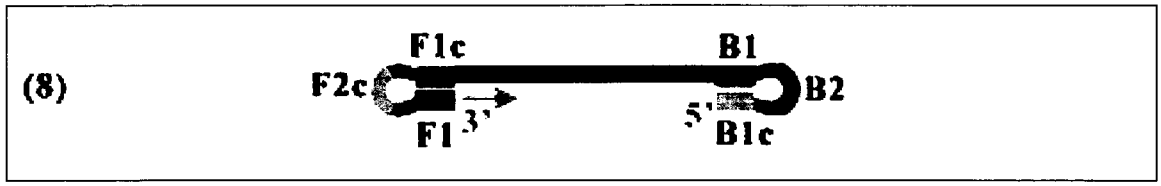


Figure 2.20 Mechanism of LAMP reaction step8.
Source: Parida M, and et al. (2008)

6.3.9) Basic principle cycling amplification step

A dumbbell-like DNA structure is quickly converted into a stem-loop DNA by self-primed DNA synthesis. FIP anneals to the single stranded region in the stem-loop DNA and primes strand displacement DNA synthesis, releasing the previously synthesized strand. This released single strand forms a stem-loop structure at the 3' end because of complementary B1c and B1 regions. Then, starting from the 3' end of the B1 region, DNA synthesis starts using self-structure as a template, and releases FIP-linked complementary strand (Step (9)). The released single strand then forms a dumbbell-like structure as both ends have complementary F1 - F1c and B1c - B1 regions, respectively (Step (11)). This structure is the 'turn over' structure of the structure formed in Step (8). Similar to the Steps from (8) to (11), structure in Step (11) leads to self-primed DNA synthesis starting from the 3' end of the B1 region. Furthermore, BIP anneals to the B2c region and primes strand

displacement DNA synthesis, releasing the B1-primed DNA strand. Accordingly, similar structures to Steps (9) and (10) as well as the same structure as Step (8) are produced. With the structure produced in Step (10), the BIP anneals to the single strand B2c region, and DNA synthesis continues by displacing double stranded DNA sequence as shown in Figure 2.21. As a result of this process, various sized structures consisting of alternately inverted repeats of the target sequence on the same strand are formed.

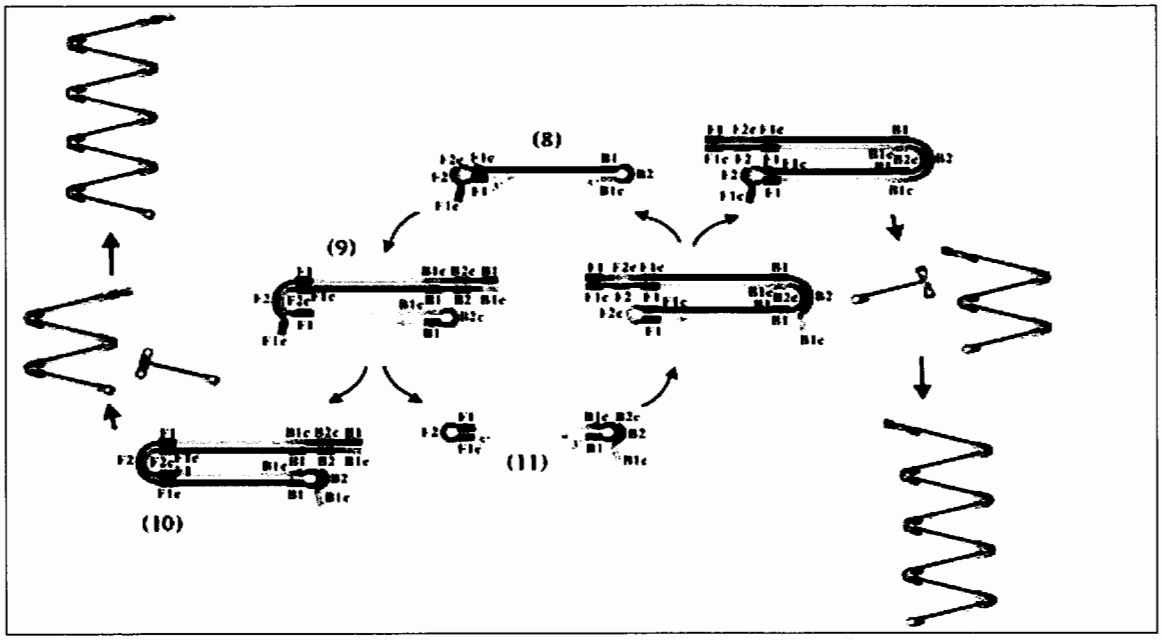


Figure 2.21 Mechanism of LAMP reaction step8-11(cycling amplification step).

Source: Parida M, and et al. (2008)

6.3.10) Principle of loop primer

The Loop Primers (either backward (B) and forward (F)), containing sequences complementary to the single stranded loop region (either between the B1 and B2 regions, or between the F1 and F2 regions) on the 5' end of the dumbbell-like structure, provide an increased number of starting points for DNA synthesis for the LAMP method. An example is shown in the Figure 2.22 where there is an amplified product containing six loops. In the original LAMP method, four of these loops would not be used, but through the use of Loop Primers, all the single stranded loops can be used as starting points for DNA synthesis (Notomi T, et al.,

2015). Many studies suggested that loop primers strongly reduce the amplification time compared to LAMP reaction without loop primers.

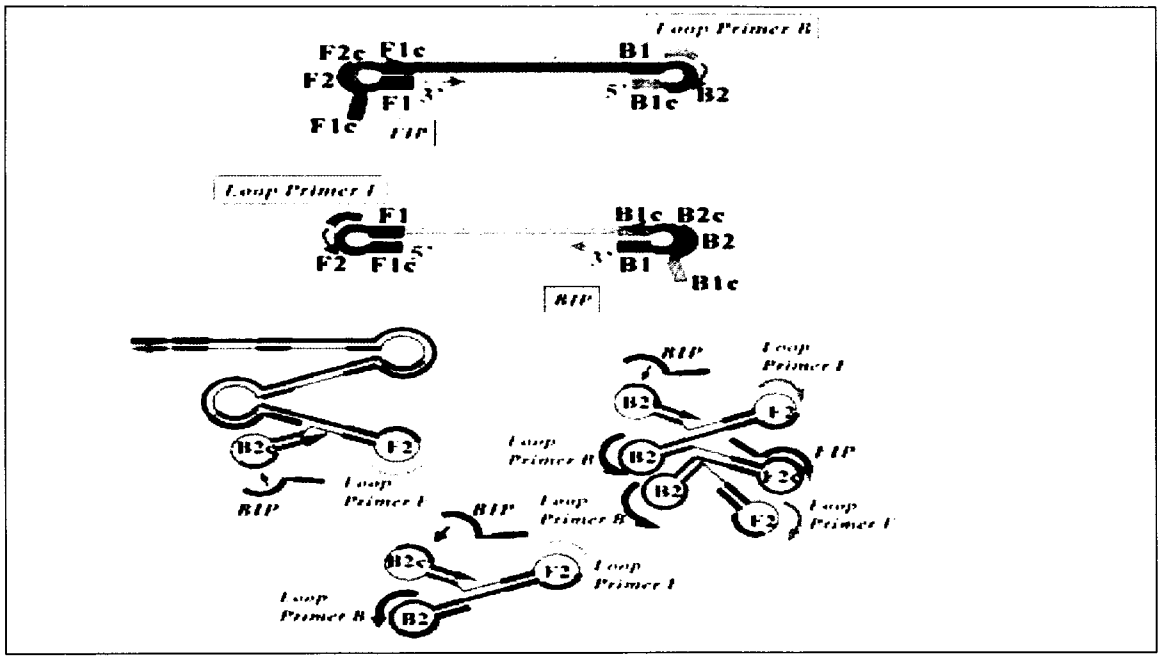


Figure 2.22 Principle of loop primers.

Source: Parida M, and et al. (2008)

2.8.2.3 Comparison of isothermal amplification methods

The isothermal amplifications methods differ from each other in terms of operating temperature, diagnostic application, mechanism, strengths and weaknesses. NASBA, RCA, and RPA reactions allow nucleic acid amplification at low temperature, thus requiring less energy to operate than higher temperature isothermal amplification (LAMP and HDA). Many miniaturized isothermal systems exploit the strand-displacement activity of a DNA polymerase (LAMP, RCA, RPA and MDA) to cyclically amplify a target in less than an hour. Target application can vary from single stranded DNA or RNA molecules to double stranded DNA samples. DNA is often preferred, where appropriate, because it is more stable than RNA and will be likely associated with a higher sensitivity in samples stored or transported in suboptimal conditions.

It should be noted that the choice of the method is strictly related to the target. NASBA for example is especially suited for RNAs such as mRNA, rRNA,

tmRNA or genomic RNA, while it cannot amplify double-stranded DNAs unless subjected to a denaturation step. On the other hand, NASBA is not free from drawbacks, since it is prone to ribonuclease (RNase) contamination and non-specific adsorption that can affect the amplification signal and the signal curve of NASBA assay.

Contrary to NASBA, LAMP enables duplex DNA amplification because, in the condition of dynamic equilibrium at 65 °C, one of the LAMP primers can anneal to the complimentary sequence of double-stranded DNA without any initial denaturation step. LAMP can also be applied to the detection of RNA with the addition of reverse transcriptase. Another important advantage of the LAMP method is its simplicity for detection of the amplification reaction, due to the insoluble pyrophosphate byproduct that can be easily quantified turbimetrically.

In a different manner, the HDA reaction exploits the activity of a DNA helicase in combination with other accessory proteins to separate complementary strands of ds DNAs, thus mimicking the denaturation mechanism in living organisms. However, HDA suffers from the limited speed and processivity of UvrD helicase that is able to amplify only short DNA sequences (from 70 to 120 bp). As an attempt to circumvent this drawback, the limited capability of UvrD HDA system in efficiently amplifying long target sequences was improved by using a thermostable UvrD helicase (Tte-UvrD).

The target applications of the RCA method are wide, ranging from small and circular single-stranded DNAs to double stranded linear DNA molecules and even mRNA. Sample pre-treatments are required before padlock probe circularization in order to allow the transformation of duplex genomic DNA into accessible single-stranded DNA targets; this additional heating step is responsible for increased power demand and more complicated control mechanisms. In mRNA detection, instead, the pre-treatment step consists of cDNA synthesis by a reverse-transcription followed by mRNA degradation by RNase H digestion. On the other hand, ss the circularizing step of a padlock probe is strictly target-dependent, high enough specificity is ensured to allow SNP analysis. Another relevant advantage of RCA is its capability to detect miRNA samples, which arises from its inherent nature for specific detection of short sequences (Zanoli LM and Spoto G, 2012).

In RPA, an initial denaturation step is not required the activity of recombinase-primer complexes that scan the double-stranded DNA by promoting the primer binding at the target sequence and the displacement of the non-template strand. The RPA reaction has the ability to proceed at a variety of temperatures since it is tolerant to fluctuations of the incubation temperature ranging from 37 °C to 42 °C. This remarkable property of RPA is of great appeal for field applications where precise temperature control is often technically challenging.

MDA exploits random exonuclease-resistant primers and the activity of φ29 DNA polymerase, already used in RCA, but to amplify linear genomic DNA rather than for circular DNA amplification. The random priming on the double-stranded DNA target is efficient enough to obtain an initial priming step that is followed by the strand-displacement process. The MDA method is capable of producing a large number of amplification products from a few DNA molecules regardless of the type of clinical sample used including buccal swabs, whole blood, finger stick blood and Guthrie cards. In addition, the use of a high-fidelity proofreading DNA polymerase ensures a significantly lower error rate during the amplification reaction and preserves DNA sequence information, an advantage to consider when an accurate genotyping is required as in whole genome amplification (WGA) (Giuffrida MC and Spoto G. 2017).

2.8.2.4 The comparison between culture, PCR and isothermal amplifications methods as listed in table 2.1

Table 2.1 Comparison of culture, PCR and isothermal amplifications methods.

Topic	Isothermal amplifications	Culture	PCR
Identification time	30 minutes -1 hour	3- 5 days	1-2 hours
Cost	Low cost	Medium	High cost
Risk of contamination	Low risk	High risk	Low risk
Confirmatory test	yes	Yes	yes
Sensitivity	High	Low	High
Specificity	High	Low	High
Applicable for Point-of-care testing	yes	No	No

2.8.2.5 Comparison of PCR and isothermal amplifications methods.

Isothermal amplification processes offer multiple advantages if compared with thermal cycling requiring methods (PCR). Isothermal DNA amplification is rapid, cost effective, easy-to-use and more tolerant to inhibitory components from a crude sample compared with PCR, which greatly simplifies point-of-care diagnosis, but at the same time showing equivalent or higher sensitivity and reliability in clinical diagnosis.

Table 2.2 Comparison of PCR and isothermal amplifications methods.

Topic	Isothermal amplifications	PCR
Amplification temperature	Constant and low	Cyclic and high
Point of care	Yes	No
Cost	Low	High
Amplification time	30 minutes-1 hour	1.5 -2 hours
Speed of amplification	High speed	Low speed
Reproducibility	Increased	Decreased
Requirement of thermocycler	No	Yes
Power consumption	Low	High
Thermal control	Reduced	Increased

Table 2.3 Comparison of PCR and LAMP.

Topic	LAMP	PCR
Amplification process	Constant	Cyclic reaction
Requirement of thermocycler	No	Yes
Number of primers	(At least) 4	2
Amplification time	30-60 minutes	> 60 minutes
Applicable for point-of- care testing	Yes	No

2.8.2.6 The detection of LAMP products.

As a powerful and novel gene amplification method, LAMP is a simple rapid diagnostic tool for early detection and identification of microbial disease including melioidosis. LAMP has integrated many innovative diagnostic detection technologies for identification pathogenic bacteria in clinical samples. LAMP uses the

Simple detection methods including: agarose gel electrophoresis, turbidity, fluorescence, DNA probes and lateral-flow dipsticks.

1) Turbidity detection method

A large amount of by-product, pyrophosphate ion, is produced; yielding white precipitate of magnesium pyrophosphate in the reaction mixture of loop mediated isothermal amplification. Figure 2.23 demonstrates the judging of presence or absence of this white precipitate allows easy distinction of whether nucleic acid was amplified by the LAMP method. An increase in the turbidity of the reaction mixture according to the production of precipitate correlates with the amount of DNA synthesized. It was observed that when nucleic acid is amplified by the LAMP method, the turbidity derived from precipitate is produced according to progress of the reaction and the turbidity can be identified by naked eye (Mori Y, et al., 2001).

Figure 2.23 Formation of turbidity in LAMP product

Source: Mori Y, et al. (2001).

2) Agarose gel electrophoresis

In LAMP, a large amount of DNA is synthesized, yielding a large pyrophosphate ion by-product. Pyrophosphate ion combines with divalent metallic ion to form an insoluble salt. Adding manganous ion and calcein, a fluorescent metal indicator, to the reaction solution allows a visualization of substantial alteration of the fluorescence during the one-step amplification reaction, which takes 30–60 min. As the signal recognition is highly sensitive, this system

enables visual discrimination of results without costly specialized equipment. The amplicons are visualized by using agarose gel electrophoresis documentation and detected under normal UV light after addition of SYBR and incubated with ethidium bromide.

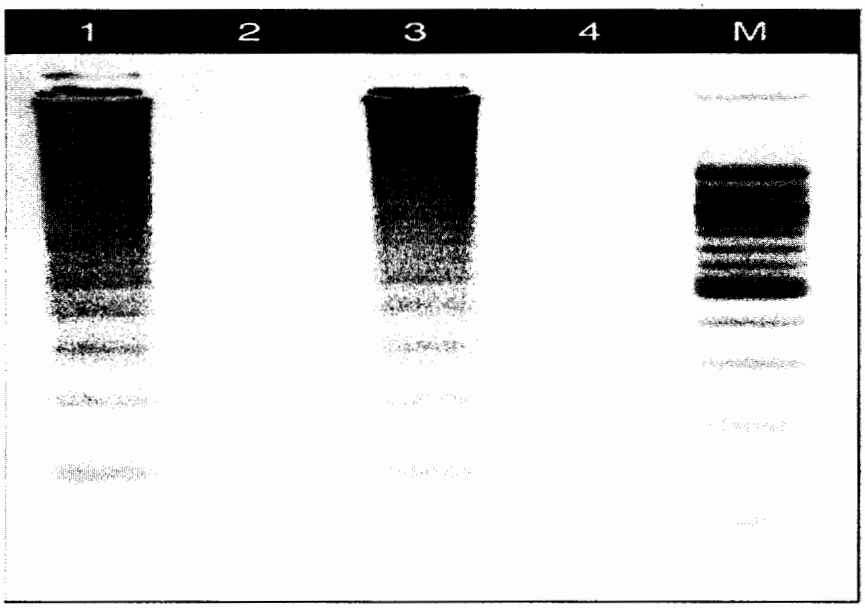


Figure 2.24 Detection of LAMP product by agarose gel electrophoresis.

Source: Tomita N, et al. (2008)

Lane 1: with target DNA, Mn2+ ion 0 mM; lane 2: without target DNA, Mn2+ ion 0 mM; lane 3: with target DNA, Mn2+ ion 0.5 mM; lane 4: without target DNA, Mn2+ ion 0.5 mM; lane M: 100-bp DNA ladder used as size maker. The characteristic ladder pattern for LAMP products was observed in lanes 1 and 3 (Tomita N, et al., 2008).

3) Fluorescent resonance energy transfer (FRET) probe technology.

One of the conventional analysis of DNA amplicons is to monitor the precipitation formed by its reaction byproduct such as magnesium pyrophosphate. The increased turbidity of the amplified products is due to the precipitation of magnesium pyrophosphate during the amplification process.

Aiming to detect the amplicons effectively and avoid the time-consuming process such as agarose gel electrophoresis, ethidium bromide staining and FRET probe technology was employed. During the process, FRET probes designed to

target loop region of the LAMP amplicons were added to conventional LAMP reagent. The mixed LAMP reaction was carried out in Light Cycler capillary tube at 90°C for 60-120min and terminated at 90°C for 2 min. The color of emitted fluorescence can be detected easily by naked eye on a conventional UV illuminator.

In conclusion, the real-time FRET assay does not require post-amplification, and products can be detected by naked eyes and fluorospectro-photometer. Generally, FRET-LAMP assay has been proved to be specific, sensitive, and accurate for detection of foodborne bacteria with minimal risks of cross contamination. However, FRET probe is difficult to be designed and the process is longer than normal LAMP assay. The assay can be monitored by naked eyes or give a semi-quantitative result through fluorospectro-photometer, and also cannot provide a quantitative analysis (Gong P, et a.,2014).

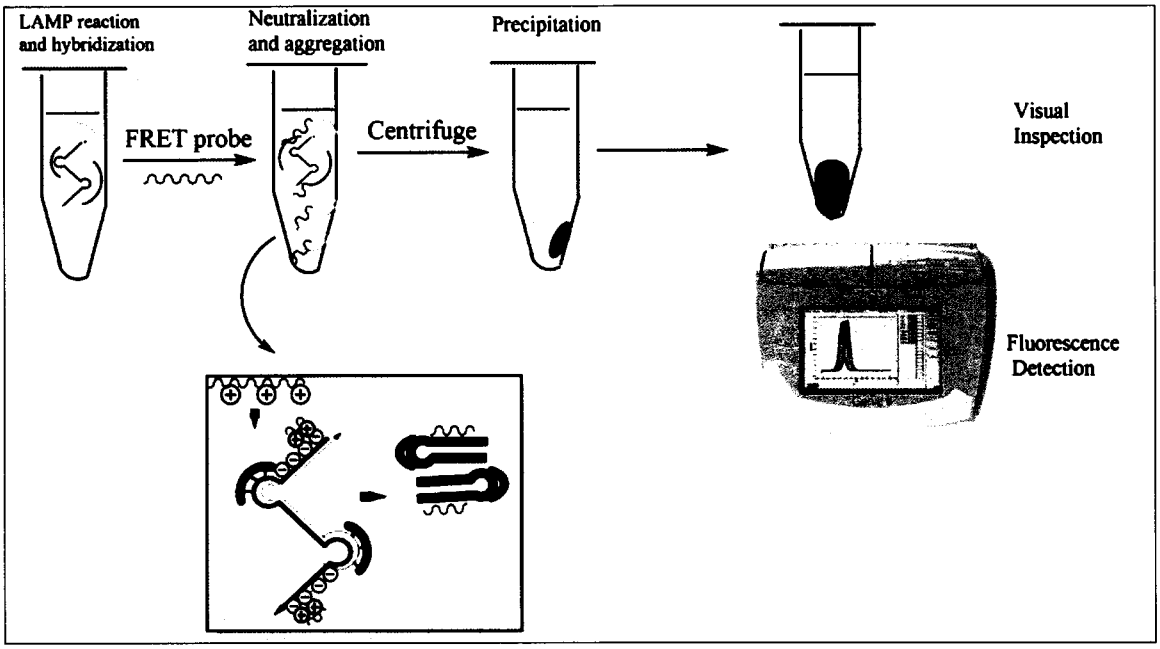


Figure 2.25 Illustration of FRET-LAMP,

Source: Gong P, et al. (2014)

The FRET probes designed to target loop region of the LAMP amplicons were added to conventional LAMP reagent, and the fluorescently labeled Oligo DNA probes hybridized to the LAMP product, detected easily by naked eye on a conventional UV illuminator. If the amplification setting is connected to a computer with the appropriate software, the results are obtained in real-time as shown in

coordinate axis. The fluorescence units are shown on the Y-axis and the time to amplification on the x-axis (Gong P, et al., 2014).

4) Detection using normal probes

Hybridization probes work in conjunction with the user's conventional PCR. Two specially designed, sequence specific oligonucleotides labelled with fluorescent dyes act in tandem as reporters to quantitate the level of the specific DNA sequence. The first oligonucleotide carries a donor fluorophore at its 3' end whereas the second oligonucleotide carries an acceptor fluorophore at its 5' end. Additionally, the second oligonucleotide is phosphorylated at its 3' end to prevent priming and hence amplification from this site. The sequences of the two oligonucleotides are selected so that they hybridize to the target/amplified DNA fragment in a contiguous arrangement such that the donor fluorophore of the first oligonucleotide is in close proximity to the acceptor fluorophore of the second oligonucleotide.

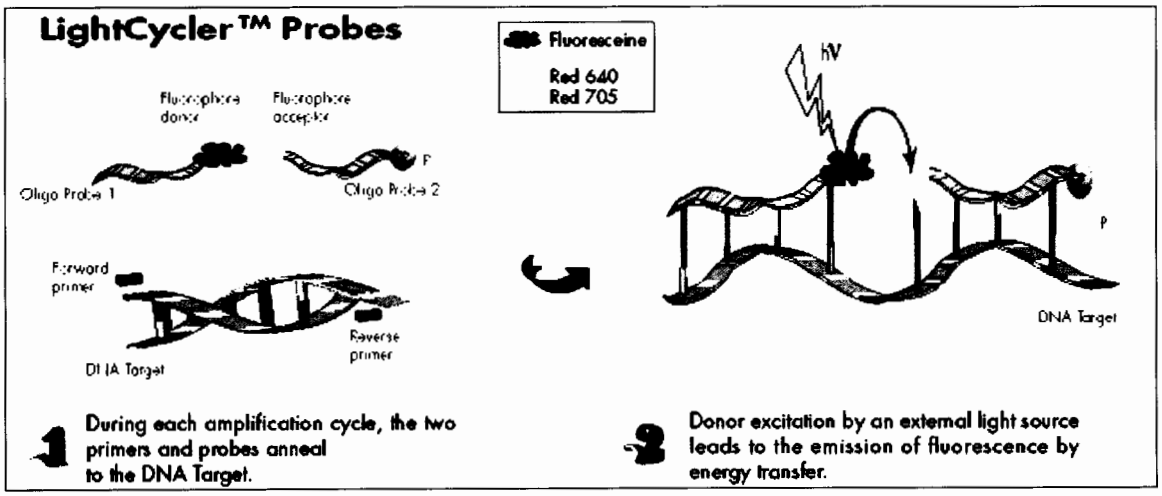


Figure 2.26 Principle of hybridization probes.

Source: Yan L, et al. (2014)

The two oligonucleotides are not in close contact, excitation from a light source (such as halogen bulb, laser or LED (Light Emitting Diode)) lead to excitation of the donor fluorphore and emission of light at a wavelength specific to the donor molecule. When the two fluorophores are in close proximity excitation of the donor fluorophore results in a transfer of energy to the acceptor fluorophore. This

transfer of energy, referred to as FRET (fluorescence resonance energy transfer), results in emission of light at a wavelength specific to the acceptor fluorophore. By measuring the signals emitted at the wavelength of the donor and acceptor fluorophore the amount of FRET can be determined. As the template/amplified target sequence is responsible for the optimal positioning the oligonucleotides the increase in amount of measured fluorescence at the acceptor emission wavelength is proportional to the increase in amount of DNA generated (Yan L, et al., 2014).

2.9 Treatment of melioidosis

Mortality in patients with B. pseudomallei infection is high and early diagnosis and specific antimicrobial therapy is necessary to reduce the fatal outcome. The antibiotic of choice for melioidosis is ceftazidime, a third-generation cephalosporin. Imipenem is considered as alternatives to ceftazidime. Since relapse rates are high, initial parenteral treatment followed by maintenance therapy with oral TMP–SMX (8–12 & 40–62 mg/kg/day) or doxycycline (4 mg/kg/day) or amoxicillin–clavulanic acid (20/5 mg/kg 8 h) for 12–20 weeks are recommended (Bearss JJ, et al., 2017).

2.10 Antibiotic resistance and susceptibility of melioidosis

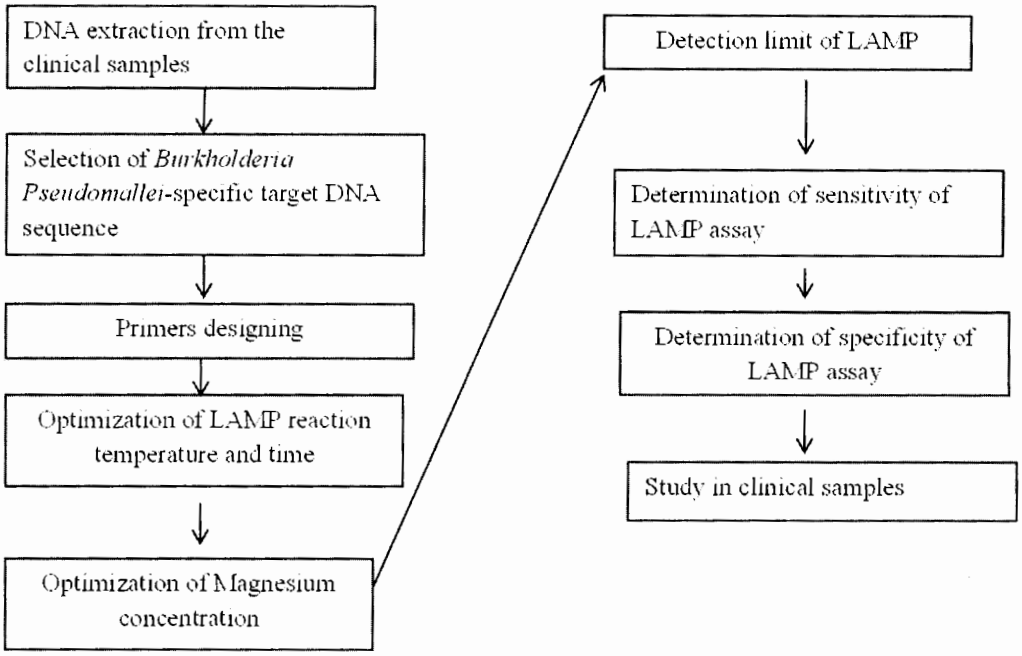
B. pseudomallei is intrinsically resistant to a wide range of antimicrobials, including gentamicin and most third-generation cephalosporins except ceftazidime. Effective parenteral antibiotics include ceftazidime and carbepenem (Teparrukkul P, et al., 2017).

B. pseudomallei often develops resistance to existing antibiotics. However, there is varying susceptibility to the various antibiotics, such as chloramphenicol, tetracyclines; trimethoprim-sulfamethoxazole, ureidopenicillins, cephalosporins, and clavulanic acid. The second important cohort of antibiotics (cephalosporins, macrolides, rifamycins, colistin, and aminoglycosides) are not effective against *B. pseudomallei*. Third-generation antibiotics, including cephalosporins, are not clinically useful in treating melioidosis; however, carbapenems and amoxicillin-clavulanate are used for treatment with broad spectrum effects. Recently, combined therapy with trimethoprim and sulphonamides was noted to decrease bacterial growth. Moreover, a treatment that combines chloramphenicol, doxycycline, and trimethoprim-

sulfamethoxazole better controls the bacterium compared with individual treatment. Fluoroquinolones have shown only weak activity during clinical trials against *B. pseudomallei*, but experimental evidence showed that it may be beneficial for immediate therapy or prophylaxis. *B. pseudomallei* develops resistance mechanisms against existing antibiotics due to enzymatic inactivation, target deletion, and efflux from the bacterium caused by chromosomally encoded genes. As a result, excessive production and mutations of the class A PenA β-lactamase can cause resistance to ceftazidime and amoxicillin-clavulanic acid. Deletion of the penicillin-binding protein-3 (PBP-3) leads to drug resistance towards ceftazidime. Similarly, over expression of the BpeEF-OprC drug-efflux pump causes resistance to trimethoprim (TMP) and TMP-sulfamethoxazole (SFZ) antibiotics (Bandeira TdJPG, et al., 2017).

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Study Design



3.2 Materials

3.2.1 Standard *B. pseudomallei*

In this study, LAMP was initially developed by using *B. pseudomallei* standard strain (ATCC 23343) which kindly provided by Melioidosis Research Center, Faculty of Medicine, Khon Kaen University, Thailand. DNA extraction was performed by a loopful culture dissolved in a microtube containing 400 µL

TE buffer (10 mM Tris/HCl (pH 8.0), 1 mM EDTA). The mixture was heated at 95°C for 10 min, centrifuged at 12000 rpm for 15 min and the supernatant was used as positive control for LAMP optimization.

3.2.2 Selection of *B. pseudomallei* specific target DNA sequence

B. pseudomallei target DNA sequence was targeted and selected in order to be used for the development of LAMP assay. The target DNA sequence (906 bp) lies from 2732831 to 2733736 which codes for outer membrane protein located at BPSS2022 gene locus (NC_006351.1). The current 'gold standard' species-specific assay for *B. pseudomallei* relies on amplification of orf2 of the type three secretion systems 1 (TTS1) cluster, which is only present in *B. pseudomallei.* TTS1gene cluster have been tested against *Burkholderia* and non-*Burkholderia* spp. strain panels including *Pseudomonas aeruginosa* and have shown promising speciation accuracy. However, although the TTS1-based assays appear to be highly reliable for identification of *B. pseudomallei,* and gives null results for other *Burkholderia* spp. that can phenotypically resemble *B. pseudomallei,* such as *B. thailandensis, B. thailandensis*-like species, B. *oklahomensis*, B. *vietnamiensis* or B. *ubonensis* (Price EP, et al., 2012).

3.2.3 Clinical samples

Ninety-one clinical samples (72 bloods, 11 pus and 8 sputum) have been collected from Sanpphasitthiprasong Hospitals, Ubon Ratchathani Province, Thailand, using appropriate samples transport media, theses samples have been used to prepare *B. pseudomallei* genomic DNA preparation. Genomic DNA was extracted using the GF-1 Bacterial DNA Extraction Kit (Vivantis®, USA). DNA concentration and quality have been verified by spectrophotometer (Thermo Scientific NanoDropm200C, USA) then the all extracted DNA samples were stored at -20°C until used.

3.3 Methods

3.3.1 LAMP Primers designing

Specific *B. pseudomallei* LAMP primers set have been designed from the published data (GenBank accession number AF332093) by using the Primer Explorer

V4, LAMP primer designing software (https://primerexplorer.jp/e/). The primer set is composed of four primers recognizing six distinct regions of the specific target DNA sequence composed by the type three secretion systems 1 (TTS1) cluster, which is only present in *B. pseudomallei.* Taking into considering that the TTS1 gene is only found in in *B. pseudomallei* it is totally clear that these primers designed for LAMP assay would not have the cross reactions with any other bacteria that might be present in clinical samples either as pathogen or non-pathogen. Forward Inner Primer (FIP) consists of the F2 region (at the 3' end) that is complementary to the F2c region, and the same sequence as the F1c region at the 5' end. Forward Outer Primer consists of the F3 region that is complementary to the F3c region.

Backward Inner Primer (BIP) consists of the B2 region (at the 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end. Backward Outer Primer consists of the B3 region that is complementary to the B3c region and two loop primers have been used to perform LAMP assay. Loop primers accelerate the amplification reaction. All primers were listed in Table 3.1.

Table 3.1 Primers designed for amplification of *B. pseudomallei* target gene.

Primer Name	Position	Primer Sequence (5'-3')	size (bp)
F3	1158-1173	GGGATGCCTCGCCAAC	16
B3	1347-1365	GCGCTATGCGGAAGTCATC	19
FIP	F1c/TTTT/F2	T ATTCGGCGTGGAGGCGAAAAA TTTTCGACATACTCCG ATGCGGC	45
BIP	B1c/TTTT/B2	CGTTGTGCAGACCCACCGATTTTT TTGAGAACTCGGCGAGTTGG	45
LF	1203-1221	TCAGGTGCTGGCTCATCAA	19
LB	1287-1306	TGCAGACGACGTCTCCACAT	20

3.3.2 Optimization of LAMP assay

LAMP conditions for temperature, time and MgSO4 concentration for *B. pseudomallei* have been optimized by amplifying 100 ng/µl of positive control. The amplification was carried out in 25 µl final volume containing a mixture of 0.4µM of each outer primer (F3, B3), 1.6 µM of each inner primer (FIP/BIP), 0.8 µM of loop primers (LF, LB), 5 mM of MgSO4, 5M of betaine, 0.4 mM of dNTPs and 8 units of Bst DNA polymerase along with 10x of supplied thermal polymer buffer and a

specified amount of double-stranded target DNA then the mixture have been incubated at 5 different reaction temperature (57, 59, 61, 63, and 65oC) then the optimal temperature have been be selected. Using the LAMP optimal temperature, MgSO4 concentration have been optimized using different concentration of MgSO4 (1, 2, 3, 4, 5, 6, 7 and 8 mM) while the concentration of other components have been remained constant. The optimal temperature and optimal MgSO4 concentration have been used to optimize reaction time, and six different incubations times including 10,20,30,40,50,and 60 minutes were evaluated to optimize the LAMP assay.

3.3.3 LAMP reaction

The LAMP reaction was carried out in a final volume of 25 µl. The reaction mixture contained 0.4 µM of each outer primer (F3, B3), 1.6 µM of each inner primer (FIP/ BIP), 0.8 µM of loop primers (LF, LB), 5mM of MgCl2, 5M of betaine, 0.4 mM of dNTPs and 8 units of Bst DNA polymerase along with 2.5 µl of 10x of supplied thermal polymer buffer and a specified amount of double-stranded target DNA. The mixture has been incubated at 63oC for 60 minutes followed by heating at 80oC for 10 minutes to terminate reaction. The LAMP products were separated on 2% agarose gel electrophoresis followed by ethidium bromide staining and detected under UV light.

3.3.4 Evaluation of analytical performance of LAMP for *B. pseudomallei* detection.

3.3.4.1 Detection limit of LAMP assay

To determine the detection limit of LAMP assay genomic DNA was serially 10-fold diluted. The reactions were performed and master mix without DNA template was added as negative control. The LAMP reaction products were visualized and detected by 2% agarose gel electrophoresis. The detection limit of LAMP assay was the lowest concentration which demonstrates positive band reaction.

3.3.4.2 Specificity

The specificity of LAMP primers for *B. pseudomallei* was examined by testing 100ng/ µl of total DNA extracted from others bacteria. LAMP reactions were carried out under the optimized conditions and the amplifications products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining and visualization by UV transilluminator. The bacteria used including *B.*

mallei, B. thailandensis, Shigella spp., *Salmonella* spp., E. coli, *Klebsiella pneumonia and P. aeruginosa.*

3.3.4.3 Comparison study in clinical samples

To evaluate LAMP assay in clinical samples, a total of 91 samples including blood, sputum and pus were firstly tested by culture. All samples were then subjected to LAMP assay. The analytical sensitivity, specificity, positive predictive value and negative predictive value of LAMP were calculated using standard formulas based on culture results as gold standard.

3.3.5 Statistical analysis

All statistical analysis was performed using SPSS software. Descriptive statistic, mean and standard deviation have been calculated to describe the data used in this study. Standards conventional bacteria culture results were used to calculate sensitivity and specificity of LAMP assay. To compare the mean difference between two independent groups, student t-test and Mann-Whitney U test were performed in case of three or more variables.

CHAPTER 4
RESULTS

4.1 Optimal conditions of LAMP for *B. pseudomallei* detection

LAMP conditions for temperature, LAMP reaction time and MgSO4 concentration for *B. pseudomallei* was optimized by amplifying 100 ng/µl of positive control and 63oC was the optimal temperature of LAMP reaction as shown in Figure 4.1

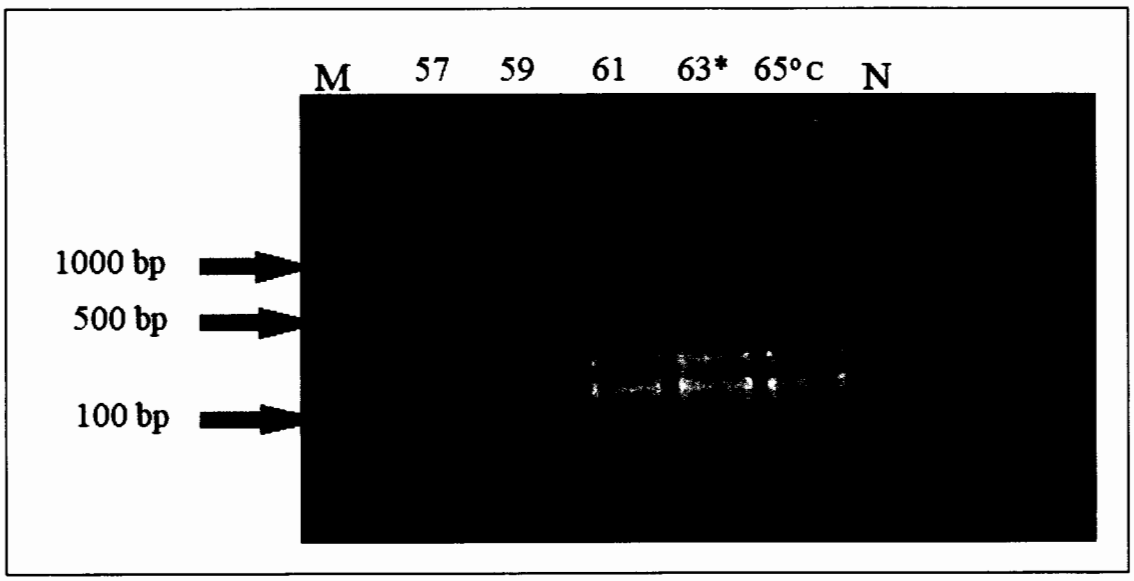


Figure 4.1 Optimal reaction temperature of LAMP assay. M: DNA Marker, N: Negative control. (* indicates the optimal temperature)

4.2 Optimization of magnesium sulfoxide concentration and reaction conditions

Using the LAMP optimal reaction temperature, MgSO4 concentration was optimized using different concentration of MgCl2 (1, 2, 3, 4, 5, 6, 7 and 8 mM) while the concentration of other components have been remained constant. After experiment, concentration of 4 mM of magnesium sulfoxide had a good reaction (as shown in Figure 4.2) and have been chosen as the optimal magnesium concentration for further studies.

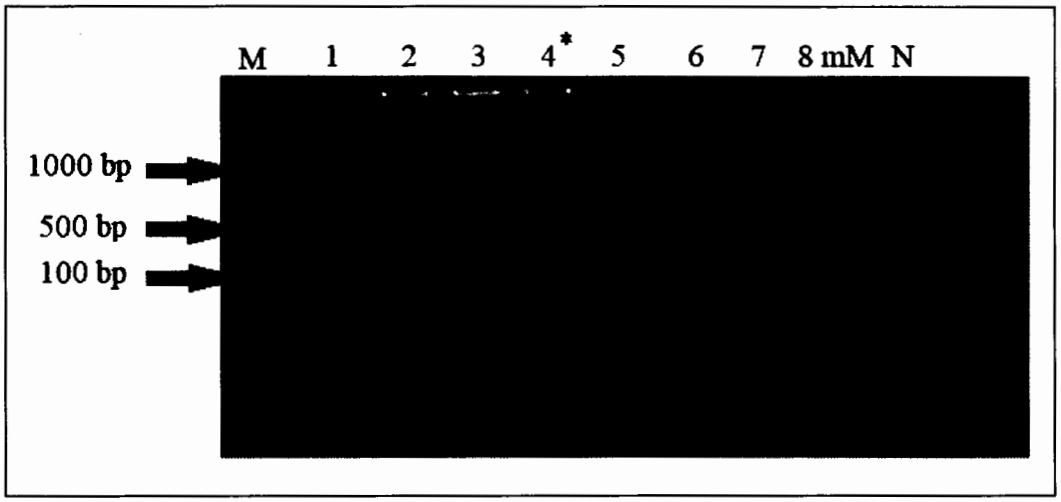


Figure 4.2 Optimal MgS2+ concentration of LAMP assay. M: DNA Marker, N: Negative control. (* indicates as optimal concentration.)

4.3 Optimization of reaction time

The optimization of reaction time was performed using the LAMP optimal temperature and magnesium concentration at different reaction time including 10, 20, 30, 40, 50 and 60 minutes while the concentration of other components have been remained constant. After experiment maximum amplification was obtained at 60 minutes (as shown on Figure 4.3); therefore the optimal reaction time for LAMP assay was established at 60 minutes and applied for all further experiments.

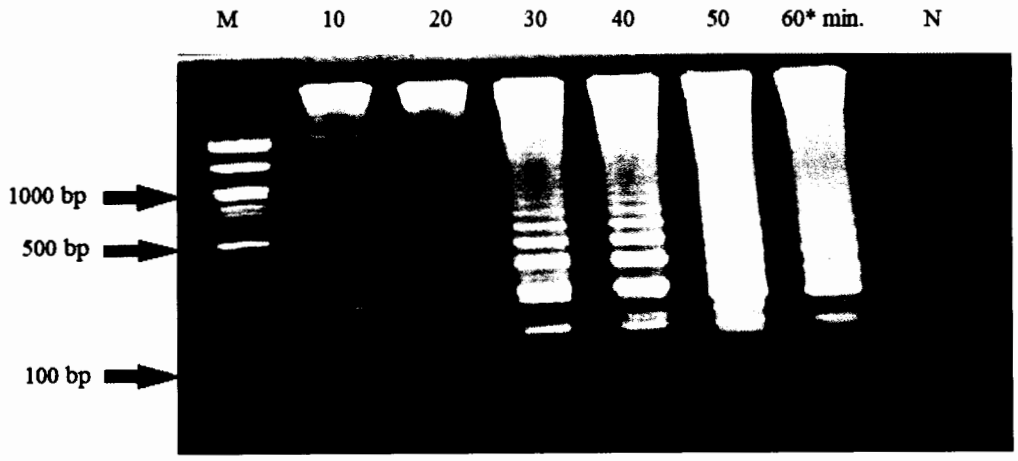


Figure 4.3 Optimal reaction time of LAMP assay. M: DNA Marker, N: Negative control. (* indicates the optimal reaction time).

4.4 analytical performance of LAMP assay for *B. pseudomallei* detection.

4.4.1 Detection limit of LAMP assay

To determine the detection limit of LAMP assay genomic DNA was serially 10-fold diluted. The LAMP reaction products were visualized and detected by agarose gel electrophoresis. The lower limit of detection of LAMP assay was 10 pg/µl as shown in Figure 4.4.

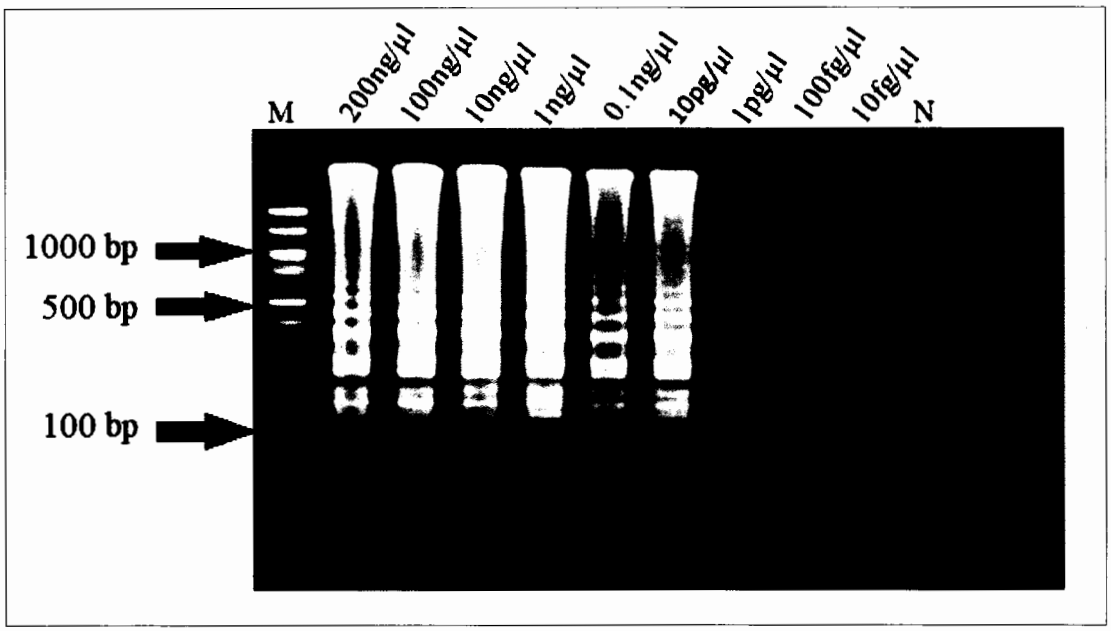


Figure 4.4 Detection limit of LAMP assay for the detection of *B. pseudomallei*

M: DNA marker, N: Negative control

4.4.2 Specificity of LAMP assay for *B. pseudomallei.*

The specificity of LAMP primers for *B. pseudomallei* was examined by testing 100 ng/µl of total DNA extracted from other bacteria. LAMP reactions were carried out under the optimized conditions and the amplifications products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining and visualization by UV transilluminator. The results showed that DNA amplification was only observed when the primer set was reacted with *B. pseudomallei* as shown in Figure 4.5. The positive reaction was clearly seen whereas no LAMP products were detected in reaction carried out with other organisms including *B. mallei, B.*

thailandensis, *Shigella* spp., *Salmonella* spp., *E. coli, Klebsiella pneumoniae, P. aeruginosa.*

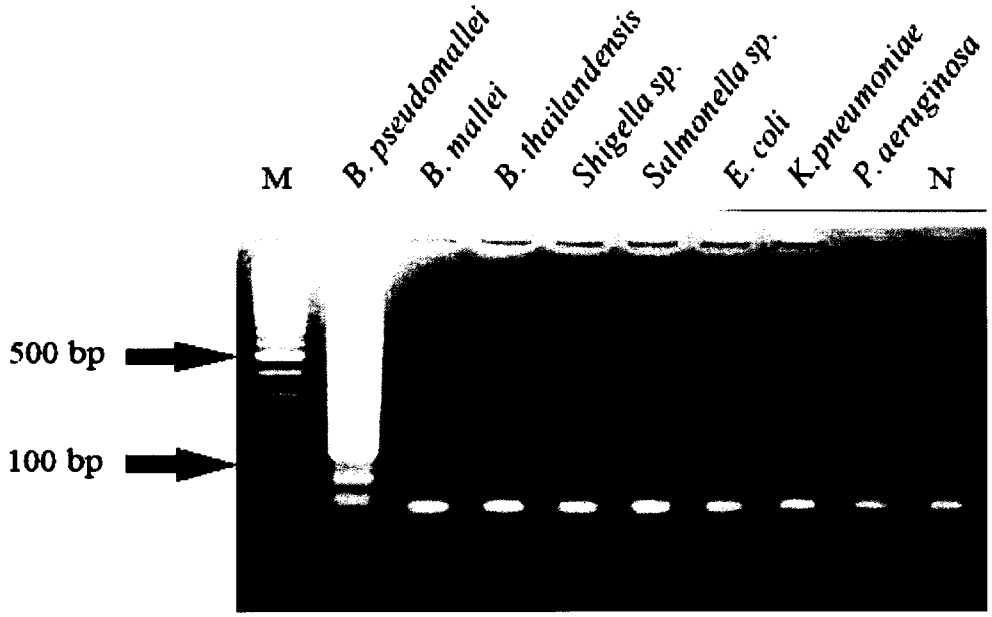


Figure 4.5 Specificity of LAMP assay for *B. pseudomallei.* M: DNA marker, N: negative control

All 18 samples cultured negative for melioidosis were negative for *B. pseudomallei* (analytical specificity, 100%) by LAMP.

Using the results of comparison of conventional culture and LAMP, the positive and negative predictive values have been calculated in order to determine the analytical performance of the LAMP assay. From this comparison, the positive predictive value was 100% and negative predictive value was 100% (Table 4.1). Therefore, the analytical performance of LAMP developed in this study is acceptable.

Table 4.1 Comparison of culture and LAMP ASSAY

		Culture (gold standard)		
		Positive	**Negative**	
LAMP	**Positive**	True positive (TP): **73**	False negative (FN): **0**	**Positive predictive value(PPV):** $\frac{\text{TP}}{\text{TP}+\text{FP}} \times 100 \Rightarrow \frac{73}{73+0} \times 100 = 100\%$
	Negative	False negative (FP) : **0**	True negative (TN): **18**	**Negative predictive value (NPV):** $\frac{\text{TN}}{\text{TN}+\text{FN}} \times 100 \Rightarrow \frac{18}{18+0} \times 100 = 100\%$
		Sensitivity: TP $\frac{TP}{TP+FN} \times 100$ $\Rightarrow \frac{73}{73+0} \times 100 = 100\%$	**Specificity:** $\frac{TN}{TN+FP} \times 100$ $\Rightarrow \frac{18}{18+0} \times 100 = 100\%$	

Table 4.2 Diagnostic performance of LAMP assay to detect *Burkholderia pseudomallei* in clinical samples

strains	Total no. of samples	Number of samples detected by LAMP (culture method)		Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
		Positive	Negative				
B. pseudomallei	73	73	0(0)	100	100	100	100
B. mallei	3	0(0)	3(3)	100	100	100	100
B. thailandensis	10	0(0)	10(10)	100	100	100	100
Other bacteria strains	5	0(0)	5(5)	100	100	100	100

CHAPTER 5
DISCUSSION AND CONCLUSION

B. pseudomallei predominantly causes melioidosis and its clinical manifestations resemble those of diseases caused by other *burkholderia* species (Hatcher CL, et al., 2015). In humans, timely diagnosis and treatment with correct antibiotics is critical as acute *B. pseudomallei* infection may lead to death in absence of appropriate antibiotic therapy.

In order to have a successful management system of melioidosis and having a full control of *B. pseudomallei* in term of the prescription of effective drugs for appropriate patients in adequate doses for treatment the person in case, for preventing the evolution of the antibiotic resistance of *B. pseudomallei,* for having an effective point of care laboratory testing applicable in resource-poor endemic areas, a good and reliable laboratory method for rapid detection of *B. pseudomallei* is required. Such detection method should play an important role in early diagnosis of this infection. LAMP represents a novel DNA amplification technique that can amplify bacterial DNA from clinical samples collected from patients infected by melioidosis.

In this study, a sensitive and specific LAMP assay was developed for detection of *B. pseudomallei* and this assay was compared to a cultural technique for diagnosis of melioidosis. TTS1 gene was selected as a LAMP assay target DNA sequence. TTS1 gene cluster has earlier been reported to be present only in *B. pseudomallei* and absent in closely related *Bulkholderia* species as well as other bacteria strains. (Dzulaikha K, et al., 2014).

The LAMP assay needs to have an optimal working temperature for amplification therefore the optimization of time of reaction was conducted using different amplification temperatures including 57, 59, 61, 63 and 65 Celcius degree. At the end there was no amplification on 57 and 59^{O}C but amplification was observed at 61, 63 and 65^{O}C and the best result was obtained at 63^{O}C and it was used for others experiments. This optimum reaction temperature of 63^{O}C was similar to others optimal LAMP temperature used in previous studies during the development and

validation of a rapid and simple fluorescence real-time loop-mediated isothermal amplification assay for detection of *Burkholderia pseudomallei* (Guo X-G, et al., 2018)

Every enzyme needs a cofactor in order to be function properly. During LAMP reaction DNA polymerase needs optimal concentration of magnesium sulfoxide to enhance the amplification therefore low concentration of magnesium sulfoxide is non effective while the high concentration can become antagonist to the amplification. As a cofactor of the LAMP reaction, magnesium sulfate oxide have been optimized using the optimal temperature optimized before while concentration of other components has been remained constant. During this optimization different concentrations of magnesium sulfate oxide have been tested but 4mM gave good reaction during the development of the LAMP assay for rapid detection of *B. pseudomallei.*

LAMP developed in this study can amplify DNA with high specificity, efficiency and rapidly under isothermal optimized conditions at 63°C for 60 minutes and is faster than PCR which require 2 to 3 hours excluding the detection step of amplified product by agarose gel electrophoresis. The optimization of time was evaluated by incubation of LAMP reaction mixture at 10, 20,30,40,50 and 60 minutes at $63^{O}C$, there was no considerable difference of positivity observed for the four different incubations reactions times including 30, 40, 50 and 60 minutes. Finally, 60 minutes was selected to be used in LAMP assay development and the optimal time found in this present study was similar to the other reported in previous study (Guo X-G, et al., 2018).

The detection limit of the LAMP assay developed in this study was 10 picograms per microliter which is a good low limit accepted for laboratory diagnostic assay. This low detection limit was similar to some other low detection limit proved in development of LAMP for diagnostic of *B. pseudomallei* conducted in Malaysia other microorganism (Dzulaikha K, et al., 2014)

There was no cross reaction with other *burkholderia* species and gram negatives bacteria meaning that no positive results were observed from the strains used during the determination of the specificity of the primers during the development of the LAMP assay. These results indicated that the LAMP assay was highly specific for *B. pseudomallei* therefore the analytical specificity of LAMP developed here was 100%, this specificity is similar to the previous findings reported for the diagnosis of *B.*

pseudomallei using PCR where the specificity was 100% (Novak RT, et al., 2006), and also has similar specificity found during the evaluation of rapid diagnostic test for the detection of *B. pseudomallei* in Lao People's Democratic Republic where the these rapid tests were having the specificity of 100% (Woods KL, et al., 2018).

The analytical sensitivity of the LAMP developed in this study was 100% which is a good characteristic for a screening laboratory test and this sensitivity is slightly similar to previous findings reported during the evaluation of rapid diagnostic tests for the detection of *B. pseudomallei* which was 99% (Woods KL, et al., 2018). However, this sensitivity is the highest than other sensitivities published in previous studies (Chantratita N, et al., 2008), therefore, further evaluation using a larger and more diverse samples is necessary in order to accurately determine the sensitivity and reliability of this LAMP method in clinical settings. The high specificity and sensitivity, the short time for analysis, the cost effectiveness, non-requirement of expensive equipment are the key advantages of LAMP assay.

The LAMP developed in this study was having the highest analytical performance for the laboratory diagnostic assay as it is indicated by the comparison of the conventional culture method which is gold standard in this study. The LAMP assay gave the same results as the conventional culture method either on the rate of positivity or on the rate of negativity. From the comparison, the positive and negative predictive values were 100% which means that the analytical performance of this LAMP is very high and acceptable for the detection of *B. pseudomallei* for the diagnosis of melioidosis.

Optimal LAMP assay developed in this research was evaluated in order to determine the efficiency for detection of *B. pseudomallei* in clinical samples. The evaluation was done on 91 clinical samples including those of culture-confirmed melioidosis and non-melioidosis. As bacterial culture method considered as gold standard test, the LAMP assay gave the same results as culture in clinical samples, there was no false negative or false positive while using LAMP assay. Thus, LAMP assay was effective in detecting *B. pseudomallei* in clinical samples.

In conclusion, LAMP technique represents a reliable alternative molecular isothermal amplification technique for the rapid diagnosis of melioidosis by the detection of *B. pseudomallei* with the advantage of field use and is fast, sensitive and

specific in different types of samples. The results of this study give evidence that this LAMP technique can be used as point of care laboratory diagnostic test and can be used also as molecular amplification technique for rapid detection of *B. pseudomallei*.

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APPENDIX

1. Table of culture and biochemical tests results

No	Blood agar	EMB	Ashdown	TSI	10% lactose	Arabi nose	Oxidase	citrate	Nitrate broth	Urease	SIM (Motile)			Methyl red/voges proskauer	Conclusion	LAMP with BP primers
											S	I	M			
1	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
2	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
3	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
4	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
5	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
6	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
7	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
8	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
9	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P

Note : G=Growth, NON-G= Non growth N=N, P= Positive , NG= No growth, EMB=Eosin Methylene Blue agar, SIM= Surfide Indole Motility, TSI: Triple Sugar Iron agar. A/A= acid/acid, KN= alkaline/no change, N/N= no change/ no change.

1. Table of culture and biochemical tests results (continued)

No	Blood agar	EMB	Ashdown	TSI	10% lactose	Arabinose	Oxidase	citrate	Nitrate broth	Urease	SIM (Motile)			Methyl red/voges proskauer	Conclusion	LAMP with BP primers
											S	I	M			
10	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
11	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
12	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
13	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
14	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
15	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
16	G	G	G	KN	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
17	G	G	G	A/A	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
18	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P

Note : G=Growth, N=N, P= Positive , NG= No growth, EMB=Eosin Methylene Blue agar, SIM= Surfide Indole Motility, TSI: Triple Sugar Iron agar.A/A= acid/acid, KN= alkaline/no change, N/N= no change/ no change.

1. Table of culture and biochemical tests results (continued)

No	Blood agar	EMB	Ashdown	TSI	10% lactose	Arabinose	Oxidase	citrate	Nitrate broth	Urease	SIM (Motile)			Methyl red/voges proskauer	Conclusion	LAMP with BP primers
											S	I	M			
19	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
20	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
21	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
22	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
23	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
24	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
25	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
26	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
27	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P

Note: G=Growth, N=N, P= Positive, NG= No growth, EMB=Eosin Methylene Blue agar, SIM= Surfide Indole Motility, TSI: Triple Sugar Iron agar. A/A= acid/acid, KN= alkaline/no change, N/N= no change/ no change.

1. Table of culture and biochemical tests results (continued)

No	Blood agar	EMB	Ashdown	TSI	10% lactose	Arabinose	Oxidase	citrate	Nitrate broth	Urease	SIM (Motile)			Methyl red/voges proskauer	Conclusion	LAMP with BP primers
											S	I	M			
28	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
29	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
30	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
31	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
32	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
33	G	G	G	K/N	N	N	P	N	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
34	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
35	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
36	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P

Note : G=Growth, N=N, P= Positive , NG= No growth, EMB=Eosin Methylene Blue agar, SIM= Surfide Indole Motility, TSI: Triple Sugar Iron agar.A/A= acid/acid, KN= alkaline/no change, N/N= no change/ no change.

1. Table of culture and biochemical tests results (continued)

No	Blood agar	EMB	Ashdown	TSI	10% lactose	Arabinose	Oxidase	citrate	Nitrate broth	Urease	SIM (Motile)			Methyl red/voges proskauer	Conclusion	LAMP with BP primers
											S	I	M			
37	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
38	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
39	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
40	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
41	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
42	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
43	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
44	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
45	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P

Note : G=G, N=N, P= Positive , NG= No G, EMB=Eosin Methylene Blue agar, SIM= Surfide Indole Motility, TSI: Triple Sugar Iron agar. A/A= acid/acid, KN= alkaline/no change, N/N= no change/ no change.

1. Table of culture and biochemical tests results (continued)

No	Blood agar	EMB	Ashdown	TSI	10% lactose	Arabinose	Oxidase	citrate	Nitrate broth	Urease	SIM (Motile)			Methyl red/voges proskauer	Conclusion	LAMP with BP primers
											S	I	M			
46	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
47	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
48	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
49	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
50	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
51	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
52	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
53	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
54	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P

Note : G=G, N=N, P= Positive , NG= No G, EMB=Eosin Methylene Blue agar, SIM= Surfide Indole Motility, TSI: Triple Sugar Iron agar. A/A= acid/acid, KN= alkaline/no change, N/N= no change/ no change.

1. Table of culture and biochemical tests results (continued)

No	Blood agar	EMB	Ashdown	TSI	10% lactose	Arabinose	Oxidase	citrate	Nitrate broth	Urease	SIM (Motile)			Methyl red/voges proskauer	Conclusion	LAMP with BP primers
											S	I	M			
55	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
56	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
57	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
58	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
59	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
60	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
61	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
62	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
63	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P

Note : G=G, N=N, P= Positive , NG= No G, EMB=Eosin Methylene Blue agar, SIM= Surfide Indole Motility, TSI: Triple Sugar Iron agar. A/A= acid/acid, KN= alkaline/no change, N/N= no change/ no change.

1. Table of culture and biochemical tests results (continued)

No	Blood agar	EMB	Ashdown	TSI	10% lactose	Arabinose	Oxidase	citrate	Nitrate broth	Urease	SIM (Motile)			Methyl red/voges proskauer	Conclusion	LAMP with BP primers
											S	I	M			
64	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
65	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
66	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
67	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
68	G	G	G	N/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
69	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
70	G	G	G	N/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
71	G	G	G	N/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
72	G	G	G	K/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P

Note : G=G, N=N, P= Positive , NG= No G, EMB=Eosin Methylene Blue agar, SIM= Surfide Indole Motility, TSI: Triple Sugar Iron agar. A/A= acid/acid, KN= alkaline/no change, N/N= no change/ no change.

1. Table of culture and biochemical tests results (continued)

No	Blood agar	EMB	Ashdown	TSI	10% lactose	Arabinose	Oxidase	citrate	Nitrate broth	Urease	SIM (Motile)			Methyl red/voges proskauer	Conclusion	LAMP with BP primers
											S	I	M			
73	G	G	G	N/N	N	N	P	P	P	N	N	N	P	N	*Burkholderia pseudomallei*	P
74	G	G	NG	K/N	N	N	P	N	N	N	N	N	N	N	*Burkholderia mallei*	N
75	G	G	NG	K/N	N	N	P	N	N	N	N	N	N	N	*Burkholderia mallei*	N
76	G	G	NG	N/N	N	P	P	P	N	N	N	N	N	N	*Burkholderia mallei*	N
77	G	G	NG	N/N	N	P	P	P	P	N	N	N	P	N	*Burkholderia thailandensis*	N
78	G	G	NG	N/N	N	P	P	P	P	N	N	N	P	N	*Burkholderia thailandensis*	N
79	G	G	NG	N/N	N	P	P	P	P	N	N	N	P	N	*Burkholderia thailandensis*	N
80	G	G	NG	N/N	N	P	P	P	P	N	N	N	P	N	*Burkholderia thailandensis*	N
81	G	G	NG	N/N	N	P	P	P	P	N	N	N	P	N	*Burkholderia thailandensis*	N

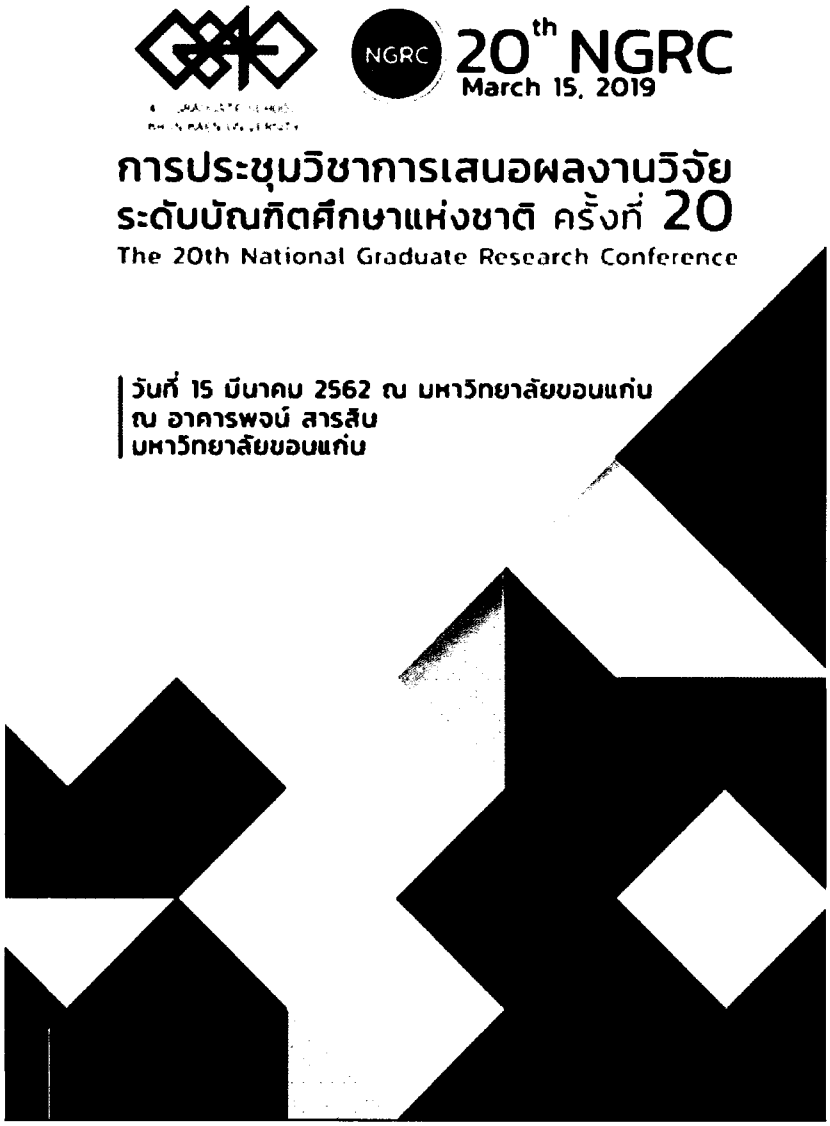
Note : G=G, N=N, P= P , NG= No G, EMB=Eosin Methylene Blue agar, SIM= Surfide Indole Motility, TSI: Triple Sugar Iron agar. A/A= acid/acid, KN= alkaline/no change, N/N= no change/ no change.

1. Table of culture and biochemical tests results (continued)

No	Blood agar	EMB	Ashdown	TSI	10% lactose	Arabi nose	Oxidase	citrate	Nitrate broth	Urease	SIM			Methyl red/voges proskauer	Conclusion	LAMP with BP primers
											S	I	M			
82	G	G	NG	N/N	N	P	P	P	P	N	N	N	P	N	*Burkholderia thailandensis*	N
83	G	G	NG	N/N	N	P	P	P	P	N	N	N	P	N	*Burkholderia thailandensis*	N
84	G	G	NG	N/N	N	P	P	P	P	N	N	N	P	N	*Burkholderia thailandensis*	N
85	G	G	NG	N/N	N	P	P	P	P	N	N	N	N	N	*Burkholderia thailandensis*	N
86	G	G	NG	N/N	N	P	P	P	P	N	N	N	N	N	*Burkholderia thailandensis*	N

Note: G=G, N=N, P= P , NG= No G, EMB=Eosin Methylene Blue agar, SIM= Surfide Indole Motility, TSI: Triple Sugar Iron agar. A/A= acid/acid, KN= alkaline/no change, N/N= no change/ no change.

5. Publication



สารบัญ

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Development of Rapid Detection for *Burkholderia pseudomallei* by Loop-mediated Isothermal Amplification

การพัฒนาตรวจหาเชื้อ Burkholderia pseudomallei อย่างรวดเร็วโดยเทคนิค loop-mediated isothermal amplification

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ABSTRACT

The present study was aimed to establish a loop-mediated isothermal amplification (LAMP) technique for the rapid detection of *Burkholderia pseudomallei*, the causative agent of melioidosis. A set of six specific primers targeting type III secretion system 1 (TTS1) were designed for the LAMP test. The reaction was optimized using different reaction temperature and Mg^{2+} concentrations. The detection limit of LAMP assay was 10 pg/μL and no cross-reaction was observed, indicating that LAMP assay has high specificity. The assay showed successful detection of *B. pseudomallei* in clinical samples. Using culture method as gold standard, the sensitivity, specificity, positive predictive values, negative predictive values of LAMP were 100%. In conclusion, LAMP assay is a high efficiency, low cost diagnostic tool, useful for rapid, accurate, direct detection of *B. pseudomallei* for clinical diagnosis.

บทคัดย่อ

ในการศึกษานี้มีวัตถุประสงค์เพื่อจัดตั้งเทคนิค loop-mediated isothermal amplification (LAMP) เพื่อตรวจหาเชื้อ *Burkholderia pseudomallei* อย่างรวดเร็ว ซึ่งเชื้อดังกล่าวเป็นสาเหตุของการเกิดโรค melioidosis ในปฏิกิริยาแลมป์ประกอบไปด้วยไพรเมอร์จำนวน 6 เส้นที่ออกแบบโดยอ้างอิงยีน type III secretion system 1 (TTS1) การศึกษาสภาวะที่เหมาะสมสำหรับปฏิกิริยาประกอบไปด้วยอุณหภูมิและความเข้มข้นของ $MgSO_4$ ที่เหมาะสม ผลการศึกษาแสดงให้เห็นว่าปฏิกิริยาแลมป์มีค่า detection limit เท่ากับ 10 pg/ μL โดยไม่พบปฏิริยาข้ามระหว่างแบคทีเรียสายพันธ์อื่น แสดงให้เห็นว่าปฏิกิริยาที่จัดตั้งขึ้นมีความไวและความจำเพาะ ในการทดสอบกับสิ่งส่งตรวจทางคลินิกโดยเปรียบเทียบผลกับการเพาะเลี้ยงเป็นวิธีมาตรฐานพบว่าเทคนิคแลมป์ที่จัดตั้งมีความไว ความจำเพาะ ค่าทำนายผลบวก ค่าทำนายผลลบ เท่ากับร้อยละ 100 ผลการศึกษานี้สรุปได้ว่าเทคนิคแลมป์เป็นวิธีที่มีประสิทธิภาพ เหมาะสมในการพัฒนาเพื่อต่อไป

Keywords: *Burkholderia pseudomallei*, LAMP

คำสำคัญ: *Burkholderia pseudomallei* แลมป์

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