DETECTION OF *BURKHOLDERIA PSEUDOMALLEI* USING CRISPR/CAS12a TECHNOLOGY

PIMPAKA RONGKRATOK

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การตรวจหาเชื้อ *Burkholderia pseudomallei* โดยการประยุกต์ใช้เทคโนโลยี CRISPR/CAS12a

นางพิมพ์ผกา รองกระโทก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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DETECTION OF *BURKHOLDERIA PSEUDOMALLEI* USING CRISPR/CAS12a TECHNOLOGY

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a master's degree.

Thesis Examining Committee

(Assoc. Prof. Dr. Pawana Panomket)
Chairperson

(Dr. Chompunoot Wangboon)
Member (Thesis Advisor)

(Dr. Mantana Jamklang)
Member (Thesis Co-Advisor)

(Dr. Theeraya Simawaranon)
Member

(Dr. Pishyaporn Sritangos)
Member

(Assoc. Prof. Dr. Yupaporn Raksakulpiwat)
Vice Rector for Academic Affairs and Quality Assurance

(Prof. Dr. Santi Maensiri)
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พิมพ์ผกา รองกระโทก : การตรวจหาเชื้อ *Burkholderia pseudomallei* โดยการประยุกต์ใช้เทคโนโลยี CRISPR/CAS12a (DETECTION OF *BURKHOLDERIA PSEUDOMALLEI* USING CRISPR/CAS12a TECHNOLOGY) อาจารย์ที่ปรึกษา : อาจารย์ ดร.ชมพูนุท วังบุญ, 51 หน้า.

คำสำคัญ: เชื้อ *Burkholderia pseudomallei* / *orf2* / *orf11*/ CRISPR/CAS12a

เชื้อ *Burkholderia pseudomallei* เป็นเชื้อสาเหตุก่อโรคติดเชื้อรุนแรง เมลิออยโดสิส การเกิดโรคนี้ในคนพบว่ามีอาการแสดงได้หลายรูปแบบตั้งแต่ไม่แสดงอาการ แสดงอาการเฉพาะที่ หรือแสดงอาการรุนแรงจากการติดเชื้อในกระแสเลือดถึงขั้นสูญเสียชีวิต อาการทางคลินิกและการตรวจหาเชื้อนี้อย่างรวดเร็วในผู้ป่วยจึงมีความจำเป็นต่อการวินิจฉัยโรคติดเชื้อนี้ การวินิจฉัยการติดเชื้อแบคทีเรียแบบดั้งเดิมจะใช้วิธีการเพาะเลี้ยงเชื้อและการทดสอบทางชีวเคมี ซึ่งต้องใช้เวลานาน นอกจากนี้ เครื่องตรวจหาเชื้ออัตโนมัติยังไม่มีการนำมาใช้ในห้องปฏิบัติการขนาดเล็ก ดังนั้นการตรวจหาเชื้อ *B. pseudomallei* ด้วยวิธีที่ง่าย สะดวก รวดเร็ว ที่มีความจำเพาะและความไวสูงจึงมีความจำเป็นอย่างมากสำหรับการวินิจฉัยโรคนี้ได้ทันท่วงที ในปัจจุบัน การใช้เทคโนโลยีคริสเพอร์แคส (CRISPR-Cas) ได้รับความสนใจเป็นอย่างมากในการนำมาประยุกต์ใช้เพื่อตรวจวินิจฉัยโรคติดเชื้อ โดยอาศัยหลักการที่ไกด์อาร์เอ็นเอ (gRNA) เข้าจับกับดีเอ็นเอเป้าหมายและกระตุ้นโปรตีนแคสซึ่งเป็นเอนไซม์ให้เกิดการตัดที่ตำแหน่งจำเพาะก่อน จากนั้นโปรตีนแคสยังถูกกระตุ้นให้เกิดการตัดดีเอ็นเอแบบไม่จำเพาะด้วย การศึกษาครั้งนี้ได้พัฒนาการตรวจหาเชื้อ *B. pseudomallei* โดยใช้เทคโนโลยีคริสเพอร์แคส และใช้เฟรมเปิดในยีน T3SS-1 (*orf2* และ *orf11*) ที่อยู่ในกลุ่มของ T3SS ซึ่งมีความจำเพาะต่อเชื้อ *B. pseudomallei* มาทำการออกแบบ gRNA ในการสกัดดีเอ็นเอจากตัวอย่างเชื้อใช้วิธีการที่แตกต่างกัน การสกัดดีเอ็นเอด้วยการต้มและไม่ตกตะกอนดีเอ็นเอเป็นวิธีการที่รวดเร็วและทำได้ง่ายที่สุด ผลการศึกษาพบว่า *orf2* และ *orf11* มีแถบดีเอ็นเอที่ถูกตัดด้วยเอนไซม์ Cas12a เกิดขึ้น จึงสรุปได้ว่า gRNA ที่ออกแบบมีความจำเพาะและส่งผลให้เกิดปฏิกิริยาการตัด DNA เป้าหมายโดยเอนไซม์ Cas12a ตามมา อีกทั้งการทดสอบกับเชื้อ *Burkholderia thailandensis* ซึ่งเป็นเชื้อที่อยู่ในระดับจีนัสเดียวกันด้วย PCR ไม่พบว่ามีแถบของ *orf2* และ *orf11* ดังนั้นจึงสรุปได้ว่ายีนที่เลือกมามีความจำเพาะต่อเชื้อ *B. pseudomallei* ซึ่งชี้ให้เห็นถึงความจำเพาะของ gRNA และเมื่อทำการทดสอบเพื่อพิสูจน์ว่ามีการตัดของเอนไซม์ Cas12a ที่ DNA เป้าหมายเกิดขึ้นจริงโดยการเติม ssDNA-FQ reporter เพื่อดูการเรืองแสงที่เกิดขึ้นหากตำแหน่ง DNA เป้าหมายมีการเข้าจับของ gRNA และ กระตุ้นให้เกิดการตัดของเอนไซม์ Cas12a ที่เป้าหมาย และสามารถตัดอย่างไม่จำเพาะกับ ssDNA-FQ ตามมาหลังจากถูกกระตุ้น ผลการศึกษาครั้งนี้พบว่า สัญญาณการเรืองแสงเพิ่มขึ้นในตัวอย่าง *B. pseudomallei* strain K96423 และ *B. pseudomallei* ที่แยกได้จากตัวอย่างทาง

คลินิก แต่ไม่พบในตัวอย่างของ *B. thailandensis* ซึ่งเป็นเชื้อที่อยู่ในระดับจีนัสเดียวกัน จึงสามารถสรุปได้ว่า gRNA ที่ทำการออกแบบใหม่จากการศึกษานี้ มีความจำเพาะต่อเชื้อ *B. pseudomallei* ซึ่งสามารถนำไปประยุกต์ใช้สำหรับการพัฒนาวิธีการตรวจวินิจฉัยโรคติดเชื้อเมลิออยด์ด้วยวิธีตรวจแบบเร็วและมีความจำเพาะได้ อีกทั้งจะช่วยอำนวยความสะดวกและช่วยให้การตรวจพบการติดเชื้อ *B. pseudomallei* ในห้องปฏิบัติการจุลชีววิทยาทางคลินิกของโรงพยาบาลชุมชนขนาดเล็กมีประสิทธิภาพมากขึ้น อย่างไรก็ตาม การทดสอบความไวในการตรวจหาเชื้อ *B. pseudomallei* ของ gRNA ทั้ง *orf2* และ *orf11* ต้องทำการศึกษาต่อไป

สาขาวิชาปรีคลินิก
ปีการศึกษา 2566

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

PIMPAKA RONGKRATOK : DETECTION OF *BURKHOLDERIA PSEUDOMALLEI* USING CRISPR/CAS12aTECHNOLOGY. THESIS ADVISOR : CHOMPUNOOT WANGBOON, Ph.D. 51 PP.

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Burkholderia pseudomallei is the etiologic pathogen of a severe infectious disease known as melioidosis. The disease in humans ranges from asymptomatic to focal infection and could be life-threatening by rapid fatal septicemia. Clinical presentation and rapid detection of the microorganism are necessary for early diagnosis of melioidosis. The conventional culture of the microbe is time-consuming and requires a full set of biochemical tests. Moreover, automatic machines for identification of the bacteria are not available in small microbiology laboratories. Hence, a simple and rapid detection with high specificity as well as sensitivity of the test is required for its early diagnosis. Currently, CRISPR-Cas12a technology is an attractive tool for infectious disease diagnostic applications. The detection relies on the target-activated nonspecific endonuclease activity of Cas12a after binding to a specific target DNA via programmable guide RNAs. In this study, we developed a new methodology for a rapid detection of *B. pseudomallei* using CRISPR-Cas12a technology. The specific markers of *B. pseudomallei*, *orf2* and *orf11* in T3SS-1, located on T3SS gene cluster were selected for gRNA design. The DNA of *B. pseudomallei* was extracted from the colony suspension by different methods and the boiling method without DNA precipitation gave the fastest and simplest methodology for DNA extraction. The results showed that the digestion reaction involving the gRNA specific for *orf2* and *orf11* generated DNA fragments. Therefore, this finding suggests that the newly designed gRNA was specific to the target genes leading to the digestion activity by Cas12a enzyme. In addition, the investigation in *Burkholderia thailandensis* which is classified in the same genus as *B. pseudomallei* did not show the *orf2* and *orf11* band when tested by PCR. Therefore, this finding suggests that the newly designed gRNAs were specific to the target DNA sequences leading to the digestion activity by Cas12a enzyme. Subsequently, the target-activated CRISPR/Cas12a cleavage activity was verified based

on signal amplification of ssDNA-FQ reporter. The result revealed that an increase of collateral cleavage activity of the FAM fluorophore from its quencher, leading to an increased generation of fluorescence signal, which was observed in the wild type *B. pseudomallei* strain K96243 and *B. pseudomallei* isolated from clinical sample but not in *B. thailandensis* which is classified in the same genus as *B. pseudomallei*. This concludes that the newly designed gRNAs of *orf2* and *orf11* in this study could specifically detect *B. pseudomallei*, but not other pathogens and can be used for the development of a rapid diagnostic tool for melioidosis. This could facilitate a clinical microbiological laboratory in small community hospitals to detect and interpret the infection from *B. pseudomallei* more efficiently. For the further study the sensitivity of gRNA of *orf2* and *orf11* for *B. pseudomallei* detection should be determined.

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Student's Signature Pimpaka Rangkratok
Advisor's Signature Chanpanat Wangboon
Coadvisor's Signature

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LIST OF ABBREVIATIONS

°C	Degree Celsius
g	Gram
h	Hour
min	Minute
mL	Milliliter
nm	Nanometer
OD	Optical density
µg/ml	Microgram per milliliter
µg	Microgram
µl	Microliter
µ	Micrometer
RT	Room temperature
RPM	Revolutions per minute
MW	Molecular Weight
pmole	Picomole
nmol	Nanomole
bp	Base pair
PCR	Polymerase chain reaction
NC	Negative Control
Temp	Temperature
ng/µl	Nanogram per milliliter
nt	Nucleotide
V	Volt

CHAPTER I

INTRODUCTION

1.1 Background / Problem

Melioidosis is a serious infectious disease with high mortality rate caused by *Burkholderia pseudomallei*, an environmental aerobic Gram-negative bacillus. The endemic area of melioidosis is the most common in Northern Australia, Southeast Asia, especially in Northeast Thailand. The infection in humans typically results from contact with contaminated environmental sources (soils and surface waters) by inoculation, ingestion, or inhalation (Wiersinga et al., 2006). Rice paddy workers are one of the highest risk occupational groups for contracting melioidosis. The clinical presentation following *B. pseudomallei* infection is broad and includes nonspecific symptoms range from skin and soft tissue abscesses to acute pneumonia and septicemia with fatal outcomes. The acute septicemic can occur following an incubation period of a few days and its clinical presentations are similar to other conditions such as malaria, enteric fever, typhus, leptospirosis making them difficult to differentiate. The chronic type may occur newly or follow the subacute type, either directly or as a recurrence after treatment. The illness can progress slowly and lasts over months to years, and is characterised by chronic abscesses, closely mimicking tuberculosis (Karunanayake, 2022) and chronic lung disease can also occur and can be difficult to distinguish from pulmonary tuberculosis. Hence, early diagnosis of *B. pseudomallei* infection can lead to timely treatment and the administration of appropriate medication, ultimately contributing to saving lives. Identification of *B. pseudomallei* from clinical specimens in a hospital laboratory is typically done through laboratory tests followed by biochemical identification. Although this technique is specific and relatively inexpensive, definitive identification of *B. pseudomallei* requires expertise and can be time-consuming (5 to 7 days). The culture method is not a perfect gold standard for detecting *B. pseudomallei* in clinical samples due to its low sensitivity and the presence of unculturable forms of the organism that have been associated

with previous antibiotic treatment in some patients (Selvam et al., 2021). To achieve early diagnosis of melioidosis, other techniques have been developed for detecting *B. pseudomallei* in clinical specimens. For instance, immunological tests such as immunofluorescent assays (IFAs) and molecular methods such as real-time PCR and loop-mediated amplification (LAMP) have been used. However, these methods require expensive instruments and professional operation, which limits their application in the field.

Recently, nucleic acid detection technology based on clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) has been developed. This technology has the advantages of being rapid, simple, and low cost. The detection relies on the target-activated nonspecific endonuclease activity of Cas12a after binding to a specific target DNA via programmable guide RNAs (Jolany Vangah et al., 2020). By combining the programmable specificity of Cas12a with a reporter molecule that is activated upon target recognition, these enzymes result in specific and sensitive indications of the presence or quantity of nucleic acid. CRISPR/Cas-based diagnostic technology has been successfully applied to detect a variety of highly pathogenic viruses, such as Zika virus (ZIKV), Dengue virus (DENV), human papillomavirus (HPV), and Avian Influenza A Virus (H7N9) (Wang et al., 2020). In this study, we will develop a rapid diagnosis tool based on a molecular detection system for *B. pseudomallei* using CRISPR-CAS12a technology. In this study we selected *orf2* and *orf11* from T3SS-1 gene clusters of T3SSs. The T3SS-1 gene cluster is present only in *B. pseudomallei* and not in avirulent *B. thailandensis*.

Furthermore, *orf2* was found to be present in *B. pseudomallei* and not in the related *B. mallei* or *B. thailandensis* while *orf11* is a specific marker for *B. pseudomallei* (Thibault et al., 2004)

1.2 Research objectives

1.2.1 To design a specific guided RNA for genes encoding components of the type three secretion systems (T3SS) of *B. pseudomallei.*

1.2.2 To validate an appropriate condition for *B. pseudomallei* DNA sample preparation.

1.2.3 To determine the specificity of the designed guided RNA using *in vitro* digestion of DNA of *B. pseudomallei* with CRISPR-Cas12a.

1.3 Research hypothesis

CRISPR-Cas12a technology incorporating the endonuclease CAS12a, and a specific guided RNA could be used for the development of a rapid diagnosis method for *B. pseudomallei* infection.

1.4 Scope and limitation of the study

In this study, we used *orf2* and *orf11* located in the T3SS of *B. pseudomallei*, the specific genes were used to design a guided RNA (gRNA) complementary to the target site as well as a 5' TTTV protospacer adjacent motif (PAM) on the DNA strand opposite the target sequence. DNA sample of *B. pseudomallei* were prepared using a modifying boiling procedure method that involved thermal lysis, and DNA precipitation and isolation. After that, the specificity of the guided RNA was determined by comparing the endonuclease CAS12a digestion reaction of *B. pseudomallei* genomic DNA with another Gram-negative bacilli (*Burkholderia thailandensis*) DNA. Finally, the sensitivity of the guided RNA was determined by varying the concentration of *B. pseudomallei* DNA.

1.5 Expected results

CRISPR-Cas12a technology could be applied for development of a diagnosis test for *B. pseudomallei* infection and provide a high sensitivity and specificity for the bacterial detection.

CHAPTER II

LITERATURE REVIEW

2.1 Melioidosis

Melioidosis is an infectious disease caused by the bacterium *Burkholderia pseudomallei* (*B. pseudomallei*). The disease is most common during the wet season or after major weather events including tropical storms and is most typically related to an inoculating injury through skin, ingestion, or inhalation of dispersed bacteria (Gassiep et al., 2020).

2.1.1 Epidemiology of Melioidosis

Approximately 46 nations have endemic melioidosis, and further 33 nations might contain endemic cases additionally, while autochthonous cases have not yet been reported in these countries (Gassiep et al., 2020). The endemic is high in Northern Australia, Southeast Asia, particularly in Thailand the incidence of melioidosis is still high in Northeast Thailand. Patients with diabetes mellitus, chronic kidney disease, binge alcohol consumption, cystic fibrosis and rice paddy workers are one of the highest risk groups for contracting melioidosis (Selvam et al., 2021). *B. pseudomallei* is found in a wide range of ecological niches, including soil and surface water, and has also been found to adhere to the roots of legumes (Duangurai et al., 2018). A study performed in Northeast Thailand between 1987 and 1991 suggested an incidence of 4.4 cases per 100,000 population per year. More recent observations demonstrated a peak incidence of 21.3 per 100,000 population in 2006 and an average of 12.7 per year for the period from 1997 to 2006 (Gassiep et al., 2020).

2.1.2 Clinical presentation

Melioidosis has been dubbed “the Great Imitator” due to the absence of a clinical syndrome and the ability to exhibit clinical manifestations that mimic other diseases, such as cancer, tuberculosis and other severe infections or pneumonia from another pathogen (Hemarajata et al., 2016). The similarity characterised to tuberculosis and leptospirosis by fever, headache, myalgia, mild or productive cough. Pulmonary melioidosis is the most common clinical presentation of melioidosis where the patient may have features mimicking tuberculosis. It varies from mild to overwhelming necrotizing pneumonia. In humans with melioidosis, the symptoms range from asymptomatic to focal infections such as pneumonia or organ abscesses and systemic diseases (Lee et al., 2010). The disease could be chronic or fulminant with rapidly fatal septicemia, which is often associated with bacterial dissemination to distant sites such as the lungs, liver, and spleen (Figure 2.1). Death can occur in infected humans within 48 h of symptom onset (Novak et al., 2006). In addition, *B. pseudomallei* can survive inside a variety of host cells can resistant to several antibiotics used in the empirical treatment of sepsis, making it difficult to treat, and currently, there is no vaccine to protect against melioidosis.

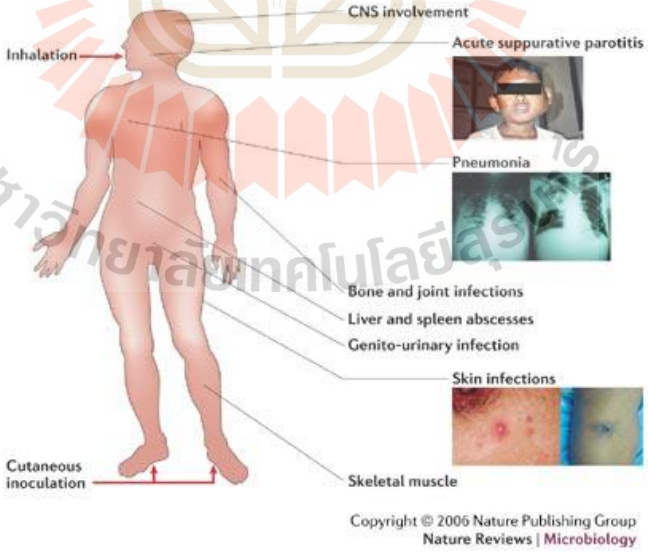

Figure 2.1 Clinical presentations of melioidosis (Wiersinga et al., 2006).

2.2 *Burkholderia pseudomallei*

B. pseudomallei was first discovered in 1911 by Whitmore and his team. This bacterium has been proven to cause melioidosis (Duangurai et al., 2018). *B. pseudomallei* is a Gram-negative, motile, environmental bacterium that appears as small Gram-negative bacilli with bipolar staining, giving them a safety pin appearance (Figure 2.2) (Gassiep et al., 2020). While the genus Burkholderia contains more than 30 species, the most pathogenic members of which are *B. pseudomallei*, *B. mallei*, *B. cepacia* complex, and *B. gladioli*, are generally recognized as human pathogens. These organisms are aerobic, non-spore-forming, nonfermenting Gram-negative bacilli. All are environmental organisms, with the exception of the host-adapted pathogen, *B. mallei*.

B. pseudomallei is found in a wide range of ecological niches, including soil and surface water, and has also been found to adhere to the roots of legumes. The wide variety of *B. pseudomallei* habitats may help explain the persistence of this bacterium in endemic areas (Duangurai et al., 2018). The routes of transmission of *B. pseudomallei* include inoculation via skin abrasion, ingestion, or inhalation of aerosolized bacteria, and ingestion. *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 are not fastidious and grow on a large variety of culture media (blood agar, MacConkey agar, EMB, etc.). Ashdown's medium is (or *Burkholderia cepacia* medium) used for selective isolation. Cultures typically become positive in 24 to 48 hours (this rapid growth rate differentiates the organism from *B. mallei*, which typically takes a minimum of 72 hours to grow). Colonies becoming dry and wrinkled after 2 days of incubation (Figure 2.3), and present a metallic appearance, and possess an earth odor.

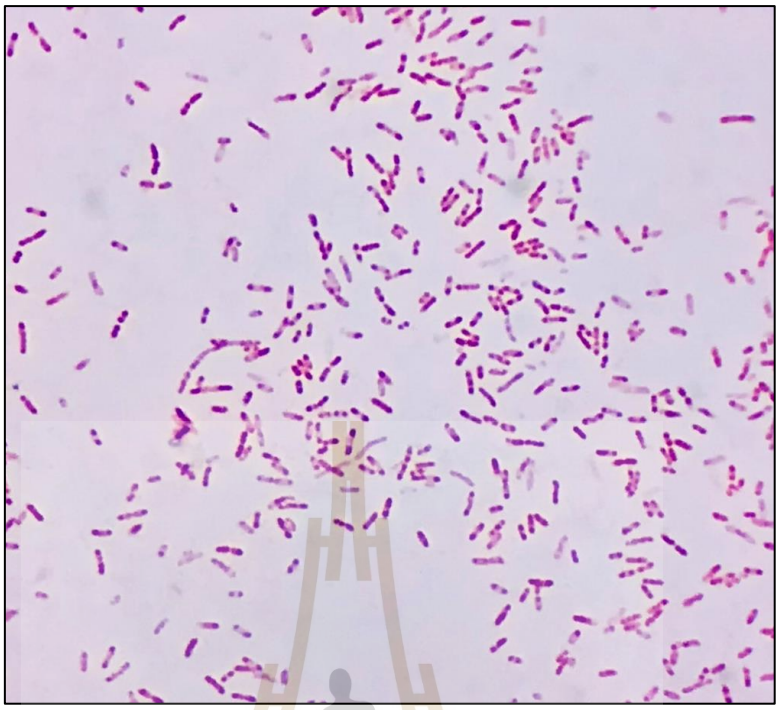

Figure 2.2 Gram stain demonstrating "safety pin" appearance of *B. pseudomallei* Magnification, x100.

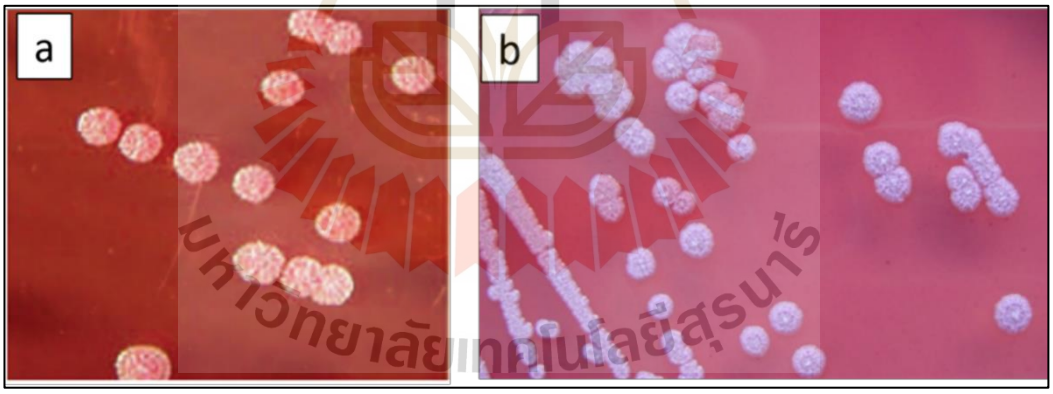

Figure 2.3 Colony morphology of *B. pseudomallei* on blood agar (a) and MacConkey agar (b) (Wiersinga et al., 2006).

2.2.1 Virulence factors of *B. pseudomallei*

B. pseudomallei has several vilurence factors including the cytotoxin *Burkholderia* lethal factor 1 (BLF1), capsular polysaccharide I, the cluster I type VI secretion system (T6SS), the Bsa type III secretion system cluster 3 (T3SS) and Type IV

pili-mediated adherence. BLF1 inhibits translation initiation and subsequent protein synthesis, translation initiation by inactivation of eukaryotic initiation translation factor 4A causing deamidation of glutamine residue (Gln-339) (Rust et al., 2018). Capsular polysaccharide I containing two separate and chemically distinct antigenic O polysaccharides against which infected patients produced antibodies. T6SS which can inject toxins and other effectors into eukaryotic cells. T3SS can secrete effector proteins into the target-cell cytosol to subvert host-cell processes. Type IV pili-mediated adherence is an important virulence mechanism mediated by carbohydrate molecules, pilus and non-pilus adhesins (Bzdyl et al., 2022).

Therefore, *B. pseudomallei* intra and intercellular life cycles can invade cells, the bacteria can invade and propagate in both phagocytic and non-phagocytic cells. These bacteria replicate intracellularly, causing lysis or spreading and infection of adjacent cells. Initially, the bacteria attach to nonphagocytic host cells via flagella, type 4 pili, and adhesins BoaA and BoaB. Cellular invasion is facilitated by the T3SS, which injects effector proteins, including BopA, BopE, BipB, BipC, and BipD. During internalization, the bacterium is enveloped by the host cell in an endocytic vesicle or endosome. Survival within the endosome occurs via multiple processes, including the production of a protease inhibitor, Ecotin. Escape from the endosome is mediated by the T3SS and subsequent upregulation of biosynthesis pathways, including purine, histidine, fatty acid, and amino acid, which aid in replication within the cytosol (Gassiep et al., 2020). Intercellular spread of *B. pseudomallei* is facilitated by membranous protrusions formed by the host cell that extend into neighbouring cells, through which *B. pseudomallei* travel by actin-mediated motility (Wiersinga et al., 2018b) *B. pseudomallei* is able to polymerize actin, spread from cell to cell, causing cell fusion and the formation of multinucleated giant cells (Figure 2.4).

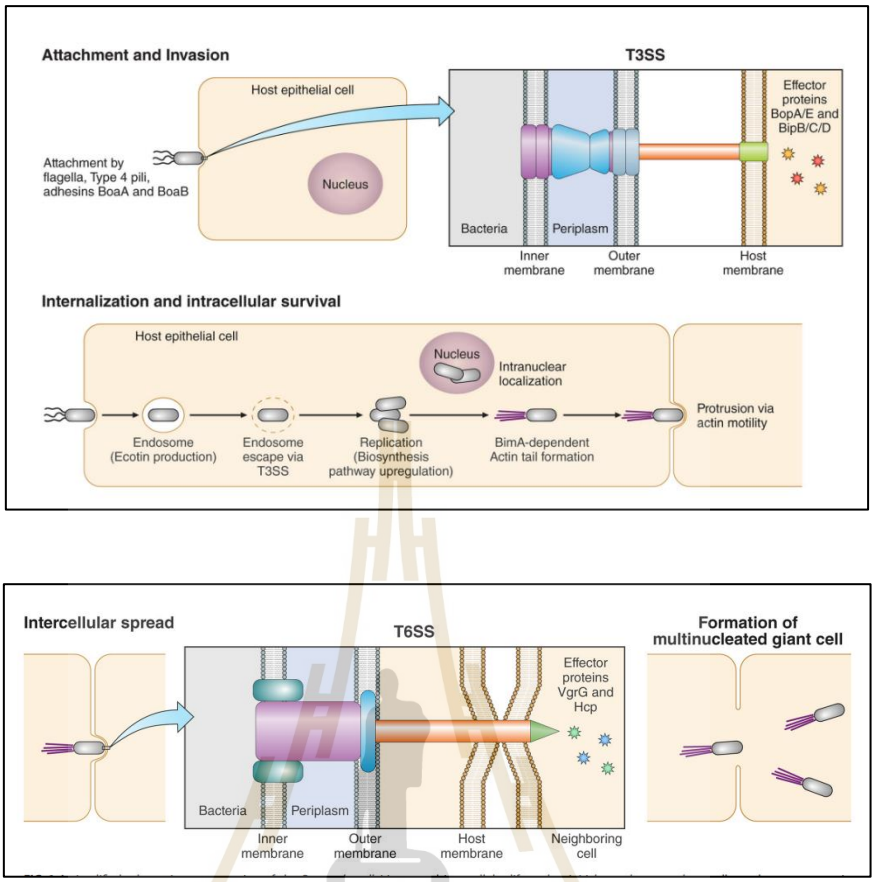

Figure 2.4 A schematic representation of the *B. pseudomallei* intra- and intercellular life cycles (Gassiep et al., 2020).

2.2.2 Type III Secretion Systems (T3SS) of *B. pseudomallei*

The versatility of *B. pseudomallei* as a pathogen is reflected in its huge 7.24 Mb genome organized into two chromosomes. One of the most important virulence factors that has been partially characterized in *B. pseudomallei* is its Type III Secretion Systems (T3SS). T3SS is documented as crucial virulence factors in numerous animal- and plant-pathogenic bacteria because it serves as a toxin delivery mechanism, enabling the pathogenic bacteria to inject toxic substance into the cytoplasm of the host cell. *B. pseudomallei* possesses three T3SS which are referred to asT3SS-1, T3SS-2, and T3SS-3. Each T3SS typically consists of a cluster of about 20 genes encoding structural components, chaperones and effectors which assemble into an apparatus resembling a molecular syringe that is inserted into the host cell membrane for the delivery of bacterial effectors into host cell cytosol (Lee et al., 2010). The genome of

B. pseudomallei consists of two circular chromosomes, with all three T3SSs residing on chromosome 2. T3SS-2 and T3SS-3 are present in the genomes of *B. mallei* and *B. thailandensis*, whereas T3SS-1 is absent from both (Vander Broek and Stevens, 2017). Furthermore, the previous study reported that T3SS-1 gene cluster encompassing part of open reading frame 2 *(orf2)* and open reading frame 11 *(orf11)* were found to be present in *B. pseudomallei* and not in the related *B. mallei* or *B. thailandensis*. This finding indicates that *orf2* and *orf11* serve as specific markers for *B. pseudomallei* and were employed in this study (Chantratita et al., 2008; Novak et al., 2006; Thibault et al., 2004). This open reading frame was later found to be present in all of a large number of Northern Australian *B. pseudomallei* strain. In the case of T3SS-3, it is required for *B. pseudomallei* to efficiently escape the endocytic vesicle and is better characterized as homologous to the Inv/Mxi-Spa secretion systems of *Salmonella spp*. and *Shigella flexneri*, respectively.

2.3 Method for diagnosis melioidosis

Conventional culture method is the routine gold standard for diagnosis melioidosis, which is time-consuming and requires 5–7 days and the culture of *B. pseudomallei* is performed only in a highly equipped biosafety level 3 laboratory (Selvam et al., 2021). In addition, this method has a limited diagnostic sensitivity, this may be because of the low *B. pseudomallei* numbers in clinical samples or the presence of unculturable forms of the organism that have been associated with previous antibiotic treatment in some patients. There are many cases that have been under/misdiagnosed as a *Pseudomonas* species because of similar colony morphology in blood agar, Gram staining and biochemical tests such as positive oxidase test. The detection of *B. pseudomallei* is difficult in routine culture media because it mimics contaminants, and the overgrowth of normal flora is observed (Selvam et al., 2021).

Molecular methods such as PCR, real-time PCR and loop-mediated isothermal amplification (LAMP) assays have prevailed for diagnosis. However, these methods require an expensive instrument and professional operation, which limits their application in the field. Currently, recombinase polymerase amplification (RPA) is

developed to detect specific DNA of *B. pseudomallei* with high sensitivity and specificity (Peng et al., 2019).

A variety of serological tests have been developed in previous years, such as the enzyme-linked immunosorbent assay (ELISA) and the indirect hemagglutination assay (IHA), and a rapid bedside immunochromatographic test. These tests are challenging for diagnosis due to a lack of international standardization and high seropositivity rates in healthy individuals. This is because of high background seropositivity in areas where the disease is endemic, combined with delayed or absent seroconversion of some patients with melioidosis. The previously study in Thai population, an IHA cutoff titer of less than 1:80 was deemed unlikely to indicate a true positive, as 21% of healthy blood donors were found to have a titer of 1:40, titers of 1:80 to 1:320 were suggestive of infection, and a titer of 1:320 was very likely to indicate infection with a specificity of 97% (Gassiep et al., 2020). Although a monoclonal antibody-based latex agglutination test and direct immunofluorescent microscopy (DIF) have been developed for use with fresh clinical specimens and fast bacterial identification after laboratory culture, respectively, neither reagent is available commercially (Chantratita et al., 2008). An alternative technique for speedy and simple DNA amplification under isothermal conditions is loop-mediated isothermal amplification (LAMP), which just needs a heat block or laboratory water bath that keeps the temperature between 60 and 65 °C constant (Chantratita et al., 2008). The serodiagnosis of melioidosis remains a challenge but still has a role to play in the diagnosis of chronic melioidosis and where culture may not always be possible, such as in neuromelioidosis or with deep-seated abscesses. Perseverance in research and development may yield a fast, easy-to-use, and cost-efficient method specifically beneficial to resource-limited settings. Hence, it is not suitable for use in the field. Therefore, a simple, rapid, accurate as well as field-applicable diagnostic method for melioidosis is urgently needed.

2.4 CRISPR/CAS: CRISPR (Clustered regularly interspaced short palindromic repeats)

CRISPR (Clustered regularly interspaced short palindromic repeats): The CRISPR-Cas system evolved as a bacterial immune system to combat the invasion of phages and other mobile genetic elements like plasmids and transposons. There are three major steps involved in the evolution of CRISPR-Cas systems in bacteria. The first step is CRISPR adaptation that during this step, foreign invader genomic fragments are integrated into a CRISPR array as spacer sequences. Subsequently, crRNA Biogenesis, the CRISPR array is transcribed into pre-crRNA, which is then processed to form mature crRNA. Finally, CRISPR interference, during this final step, the crRNA effector complexes play a crucial role in defending against invading genetic elements. These crRNAs integrate with Cas effector proteins to create crRNA effector complexes. These programmed effector complexes identify and catalyze sequence-specific destruction of foreign invading genomic fragments.

CRISPR was first discovered in the 1980s and has become the tool of choice for genome editing. Currently, all identified CRISPR/Cas systems are classified into two main classes that are further subdivided into different types and subtypes based on the organization of their loci and signature proteins. Class I CRISPR/Cas systems include type I, III, and IV, which employ multi-subunit effector complexes. Conversely, Class II CRISPR/Cas systems use a single RNA-guided, multi-domain Cas proteins to recognize and cleave target sequences. Class II CRISPR/Cas systems encompass multiple types, including type II systems such as Cas9, type V, including subtypes Cas12 and Cas14 (designated now as Cas12f), and type VI, including Cas13 systems. After recognition of the target sequence guided by a single guide RNA, (sgRNA), a CRISPR-associated nuclease (Cas) cleaves the target DNA, creating a site-specific DNA double-strand break (DSB). The structure of the sgRNA scaffold depends on the Cas protein used. CRISPR/Cas9 is most widely used for genome engineering applications. However, different Cas enzymes have different activities that can be advantageous for diagnostic applications. CRISPR/Cas12a produces staggered-end DSBs, CRISPR/Cas13 targets single-stranded RNA (ssRNA), and CRISPR/Cas14 targets single-stranded DNA (ssDNA). Interestingly, following recognition and cleavage of the specific target, Cas12a, Cas13,

and Cas14 exhibit collateral, non-specific activities against ssDNA or ssRNA. These activities can be utilized for nucleic acid detection applications (Aman et al., 2020).

Recently, next-generation molecular diagnostics technology RNA-guided CRISPR/Cas nuclease-based nucleic acid detection has been developed and demonstrate high sensitivity, specificity and reliability. CRISPR/Cas technology has been successfully used for pathogenic nucleic acid detection such as viral infection; Zika virus , Dengue virus, human papillomavirus, Avian Influenza A Virus (H7N9) and SAR-CoV2 (Broughton et al., 2020; Wang et al., 2020). The detection relies on the target-activated nonspecific endonuclease activity of Cas13 or Cas12 after binding to a specific target RNA or DNA via programmable guide RNAs. By combining the programmable specificity of Cas12/13 with a reporter molecule that is activated upon target recognition, these enzymes result in specific and sensitive indications of the presence or quantity of nucleic acid (Ding et al., 2020; Wang et al., 2020).

2.4.1 CRISPR/Cas12a

Type V CRISPR/Cas system (comprised of subtypes V-A and V-B) is also known as Cpf1 (type V-A) or C2c1 (type V-B). The system was identified in organisms such as *Francisella novicida*, *Acidaminococcus* sp., *Lachnospiraceae* sp., *Prevotella* sp. which the enzyme is in size of ~1,100–1,300 amino acids. The total guide length for Cas12a is 42-44 nucleotides (nt) with the first 19-21 nt corresponding to the repeat sequence and the remaining 23-25 nt to the spacer sequence. The endonuclease produces a staggered cut on a PAM (region of 5-TTTV) distal site on the DNA with a 5 nt overhang on the target strand, and the PAM distal end of the cleaved product is then released from the complex.

Cas12 processes its own guide RNAs, leading to increased multiplexing ability. Cas12 has also been engineered as a platform for epigenome editing, and it was recently discovered that Cas12a can indiscriminately chop up ssDNA once activated by a target DNA molecule matching its spacer sequence. This property makes Cas12a a powerful tool for detecting the target DNA in a mixture. CRISPR/Cas12a (Cpf1) proteins are RNA-guided DNA targeting enzymes that bind and cut DNA as components of bacterial adaptive immune systems. It can be used as a powerful genome editing tool

based on its ability to induce genetic changes in cells at sites of double-stranded DNA (dsDNA) cuts. This target-activated non-specific ssDNase activity, catalyzed by the same active site responsible for site-specific dsDNA cutting, is also a fundamental property of other type V CRISPR-Cas12 enzymes. Activation of ssDNA cutting requires faithful recognition of a DNA target sequence matching the 20-nucleotide guide RNA sequence can distinguishing closely related DNA sequences. Following recognition of the target sequence, Cas12a exhibit collateral non-specific catalytic activities that can be employed for nucleic acid detection, for example by degradation of a labeled nucleic acid to produce a fluorescent signal (Aman et al., 2020).

Cas12a (Cpf1), derived from *Acidaminococcus sp.* recognizes the PAM sequence 5′ -TTTV-3′ (where V represents A, G, or C) the TTTV PAM site is on the strand opposite to the targeted strand which it employs only a CRISPR RNA (crRNA) or guided RNA (gRNA), which functions as the guide RNA, directing Cas12a to the target sequence. Unlike other systems, it does not rely on a trans-activating CRISPR RNA (Figure 5). Cas12a with a bound gRNA targeting a genomic site creates a double strand cut with staggered ends.

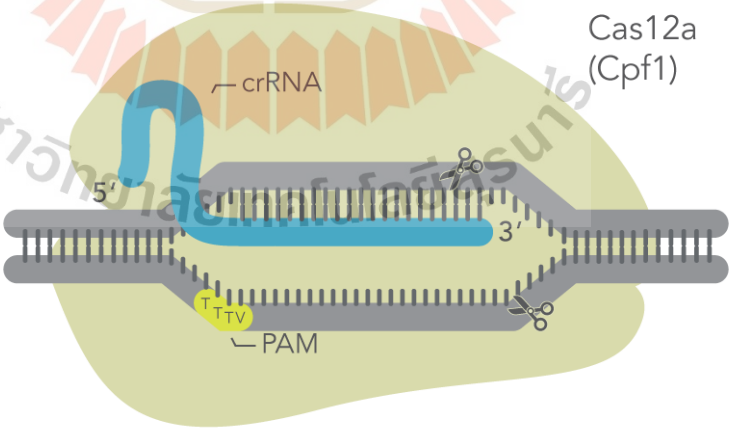

Figure 2.5 Cas12a (Cpf1) with a bound gRNA targeting a genomic site recognize the PAM sequence 5′ -TTTV-3′.

In previous study of detection of *B. pseudomallei* using CRISPR/Cas12a based on specific sequence tags demonstrated the specifically identify *B. pseudomallei* in less than 40 min. In addition, the dual-target RPA-CRISPR/Cas12a assay showed a high sensitivity as this assay can detect approximately 0.2 copies/reaction and 10 fg genomic DNA for LC1 and 2 copies/reaction and 20 fg genomic DNA for LC2 (Zhang et al., 2023). Recently, Wongpalee and cowoker reported the highly specific and sensitive of *B. pseudomallei* genomic DNA detection using CRISPR/Cas12a that crBP34-based detection assay could detect as few as 40 copies of *B. pseudomallei* genomic DNA while discriminating against other tested common pathogens. Moreover, genomic DNA of *B. pseudomallei* detection by CRISPR/Cas12a coupled with a lateral flow dipstick that the assay readout simply performed without the loss of sensitivity and does not require expensive equipment (Wongpalee et al., 2022).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial strains

The clinical isolates of *B. pseudomallei* were obtained from Chaophaya Abhaibhubejhr Hospital. The wild-type *B. pseudomallei* strain K96243 and *B. thailandensis* were obtained from the Faculty of Medicine, Khon Kean University. The isolates of *B. pseudomallei*, *B. pseudomallei* strain K96243 and *B. thailandensis* were inoculated on blood agar or MacConkey agar and incubated at 37 °C for 48 h. The bacteria were transferred to a sterile cryotube containing 60% nutrient broth and 40% sterile glycerol and then stored at -80 °C until proceeded to the next step.

3.1.2 Culture media

Blood agars in this study were prepared using tryptic soy agar 40 g in DI water 1,000 ml. The mixture then was boiled while stirring to fully dissolve all components. After that the dissolved mixture was autoclaved at 121 °C, 15 psi for 15 min and then cooled it in the water bath until the temperature down to 50-55 °C but not solidify. Adding 50 ml human blood to the cooled agar and mixed well. Pouring the agar into sterile Petri dishes and allowed it to solidify. Sterility of blood agar was checked by incubating the agar plates at 37 °C for 24-48 h and observing for any bacterial growth. Apart from sterility, hemolysis of prepared blood agars was observed by examining colonies of *Staphylococcus haemolyticus* strain, isolated from dairy cattle milk, grown on the agars.

MacConkey agars were prepared using 55 g MacConkey agar powder dissolved in 1,000 ml DI water and then gently heated to dissolve the medium completely. After that the mixture was sterilized by autoclaving at 121 °C, 15 psi for 15 min and then cooled to 45-50 °C prior dispense. The ingredients of MacConkey agars per 1 lite

composed of peptic digest animal tissue 20 g, agar 20 g, lactose 10 g, sodium taurocholate 5 g and neutral red 0.04 g (pH 7.2-7.4 at 25 °C).

Nutrient broth was prepared to stock bacteria in glycerol using 13 g nutrient broth powder dissolved in 1,000 ml DI water then the mixture was sterilized by autoclaving at 121 °C, 15 psi for 15 min. The ingredients of nutrient agar per 1 liter composed of peptone 5 g, sodium chloride 5 g, HM peptone B 1.50 g and yeast extract 1.50 g. The medium pH was adjusted to 7.2-7.4 at 25 °C.

3.1.3 Chemicals and Reagents

Glycerol purchased from Loba Chemie PVT.ltd, India was used for bacterial stock stored in – 80 °C.

Nuclease-free water obtained from Cytiva, USA was used for genomic DNA extraction, PCR and CRISPR/Cas12a system.

The GoTaq® Hot Start Colorless Master Mix from Promega, USA was used for amplification of *orf2* and *orf11* genes of *B. pseudomallei*.

Tris-borate-EDTA (TBE) buffer was prepared for running of agarose gel electrophoresis. The working concentration is 1X that contained 89 mM Tris-HCl (pH 8.0), 89 mM boric acid and 2.5 mM EDTA.

25 and 50 bp DNA ladders were used as DNA marker and DNA was stained using Visafe Green Gel stain. They were purchased from Vivantis, Malaysia.

Loading dye purchased from Biotechrabbit GmbH, Germany was used for gel electrophoresis.

The guided RNAs (gRNA) of *B. pseudomallei* designed in this study were used in CRISPR/cas12a system synthesized by Integrated DNA Technology, USA.

Lba Cas12a (Cpf1) was used for *In vitro* digestion of *B. pseudomallei* DNA target in CRISPR/cas12a system obtained from (New England BioLabs, USA).

The ssDNA-FQ reporter was used to follow the reaction of CRISPR/Cas12a system synthesized by Humanizing Genomics macrogen, South Korea.

3.2 Methods

3.2.1 Sample preparation

The wild-type *B. pseudomallei* strain K96243 and isolates of *B. pseudomallei* from clinical samples kept in 40% of sterile glycerol at -80 °C were inoculated on blood agar or MacConkey agar and incubated at 37 °C for 48 h. The bacteria were used for DNA extraction.

3.2.2 DNA extraction of *B. pseudomallei* by boiling method

In this study DNA of *B. pseudomallei* strain K96243 and isolates of *B. pseudomallei* were extracted using modification boiling method according to Armed and coworkers, 2017. The bacterial colonies of *B. pseudomallei* from the medium culture were placed into a tube containing 1,000 µl of nuclease-free water and then mixed well with a vortex mixer. After that, two aliquots of 100 µl of the cell suspension were transferred to the new microtubes and then subjected to boiling at 100 °C for 5 min. The mixtures of the two aliquots were then centrifuged at 3,000 g for 10 min at room temperature (RT). The supernatants containing DNA of the two aliquots were transferred to the separate tubes. One tube was used for PCR without precipitation while another tube was precipitated using cold absolute ethanol prior to PCR. Cold absolute ethanol was added 2 times of volume of aqueous phase to precipitate DNA at RT for 30 min and then centrifuged at 14,000 g for 5 min at RT. The supernatant was discarded, and the pellet was washed in 70% cold ethanol at 14,000 g for 5 min at RT then after further centrifugation the ethanol was removed, and the DNA pellet was allowed to dry before being resuspended in 30 µl nuclease-free water**.** The concentration, yield, and purity of DNA were determined by a NanoDrop (Thermo Fisher Scientific, DE, USA) and kept at −20 °C until proceeded as the template for polymerase chain reaction (PCR) analyses**.**

3.2.3 Selection of specific *B. pseudomallei* genes and gRNA design

The specific genes for *B. pseudomallei* were selected for a guided RNA design. The target genes of this study are located in the T3SS gene cluster of *B. pseudomallei* in which these genes encode a toxin delivery mechanism allowing pathogenic bacteria to inject toxic substances into the cytoplasm of the host's cells. The open reading

frames: *orf2* and *orf11* from T3SS-1 were reported as specific markers for *B. pseudomallei* that were used in this study (Chantratita et al., 2008; Novak et al., 2006; Thibault et al., 2004). The sequences of *orf2* and *orf11* were obtained from the completed *B. pseudomallei* K96243 genome sequence at the GenBank accession number AF074878 deposited by Winstanley et al (Winstanley et al., 1999). Sequence specificity was checked by BLAST searches for nearly exact matches via the site http://www.ncbi.nlm.nih.gov/BLAST/.

Single nucleotide gRNA for *orf2* and *orf11* were designed to be complementary to the target site as well as a 5′ TTTV protospacer adjacent motif (PAM) on the DNA strand opposite the target. The sequence of designed gRNA of *orf2* and *orf11* are shown in Table 3.1.

Table 3.1 The sequence of specific gRNA of *orf2* and *orf11* of T3SS-1 of *B. pseudomallei*.

Target site	Sequence of gRNA	Size of bp
orf2	GAUAUCCAUAGGAUCGUCGC	20
orf11	CGAAUAACGGGUAUGGGGAA	20

3.2.4 Detection of *orf2* and *orf11* of T3SS-1 of *B. pseudomallei* by polymerase chain reaction (PCR) amplification

PCR reactions were performed to detect *orf2* and *orf11* of *B. pseudomallei* situated within the gene cluster encoding T3SS-1. The primers were designed from the published sequence of strain K96243 at the GenBank accession number AF074878 by using The Primer-BLAST software, NCBI. The primers used in this study are listed in Table 3.2. Briefly, in each PCR reaction, a total volume of 50 µl contained 25 µl of GoTaq® Hot Start Colorless Master Mix (Promega, Madison, WI, USA), 2 µl of genomic DNA as template, and 10 µM of each forward and reverse primer. The BIO-RAD T100 thermal Cycler obtained from ICON@IBP Tower, Singapore is the instrument used to amplify DNA. Initially, the samples were subjected to gradient PCR (55–65 °C) to optimize the annealing temperature for each primer. The amplification steps were 1 cycle of initial denaturation at 95 °C for 2 min, 30 amplification cycles with

denaturation at 95 ℃ for 30 s, annealing at 55 ℃ (*orf11*) or 61.4 ℃ (*orf2*) for 45 s and elongation at 72 ℃ for 20 s, and the last step was followed by a final extension at 72 ℃ for 5 min. Nuclease-free water was used as a negative control for PCR. The PCR products were electrophoresed through 2% agarose gel containing ViSafe Green Gel Stain (Vivantis, Malaysia) using 1X TBE buffer at 100 Volt (V) for 45 min. Finally, the gel was visualized and photographed under ultraviolet light by Gel Doc Vilber, France.

Table 3.2 Forward and reverse primers used for detection of *orf2* and *orf11* genes of *B. pseudomallei* and PCR product size.

Target site	Oligonucleotide sequence (5′ ⟶ 3′)	PCR product size (bp)
orf2	Forward: 5′ CTCACTTCGAAGCCGAACC 3′	250 bp
	Reverse: 5′ AGTCCGAACATCTCGCTCTC3′	
orf11	Forward: 5′AAGCGTAGGCGAAACACTGA3′	335 bp
	Reverse: 5′ACGATGCGGTCAAAAGGAGT3′	

3.2.5 In vitro digestion activity of Lba Cas12a (Cpf1) on *B. pseudomallei* DNA target

For testing the specificity of the designed gRNA to *B. pseudomallei* DNA target, this study used EnGen® Lba Cas12a (Cpf1) (New England BioLabs, USA) for digestion reaction. All components were pre-incubated at room temperarture (RT) for 10 min prior to adding the PCR product of *orf2* and *orf11* at the concentration of 439 ng/ µl. Briefly, the digestion reaction was performed in the total volume of 20 µl mixed with the following components: 439 ng/µl of *orf2* or *orf11* PCR product of *B. pseudomallei* containing the target sequence, 6 µl of 300 nM gRNA containing the target sequence in the region of interest, 2 µl of 1 µM Cas12a, 2 µl of 10X reaction buffer, and nuclease-free water. The two control samples consisted of all components without DNA and another sample containing all components except gRNA. After that, the mixture was incubated at 37 ℃ for 30 min and the digestion results were electrophoresed through 2.5 % agarose gel containing ViSafe Green Gel Stain (Vivantis,

Malaysia) using 1X TBE buffer at 100 V for 45 min. Finally, the gel was visualized and photographed under ultraviolet light by Gel Doc (Vilber, France).

3.2.6 Measurement the signal of single stranded DNA fluorophore-quencher (ssDNA-FQ reporter) of FAM-BHQ1 in CRISPR/Cas12a system

The digestion reaction from CRISPR/Cas12a system at the DNA target from *B. pseudomallei* was detected using ssDNA-FQ reporter molecules. The ssDNA-FQ reporter was synthesized with a fluorescein reporter molecule attached at 5′ end and black hole quencher 1 on the 3′ end as shown in Table 3.3. The sequence of ssDNA-FQ reporter is shown in Table 3.3. The following reaction of CRISPR/Cas12a system with ssDNA-FQ reporter was performed in the total volume of 80 µl containing 8,000 ng of DNA template from PCR product *of orf2* or *orf11* of *B. pseudomallei* containing the target sequence, 6 µl of gRNA (300 nM), 1 µl of Cas12a (1 µM), 8 µl of 10X reaction buffer, 0.5 µl of ssDNA-FQ (10 µM) reporter and nuclease-free water. The control samples consisted of nuclease-free water containing ssDNA-FQ and another sample containing all components except DNA. The FAM displays excitation and emission spectrum peak wavelengths of 495 nm and 520 nm, respectively. The signals of fluorescence of cleavage products were measured every single min until 2 h at RT by fluorescence plate reader (Thermo varioskan LUX) as well as the control samples. The positive signal of fluorescence indicates that CRISPR/Cas12a specifically reacted with *B. pseudomallei* DNA target as well as collateral cleavage nearby ssDNA-FQ reporters.

Table 3.3 The sequence of ssDNA-FQ reporter used in CRISPR/Cas12a system.

Reporter	Sequence (5′ ⟶ 3′)
ssDNA-FQ	FAM-TTATTATT-BHQ1

3.2.7 Determination the specificity of selected target site *orf2* and *orf11* from T3SS-1 of *B. pseudomallei* with another Gram-negative bacilli (*B. thailandensis*)

Another closely related Gram-negative bacilli of *B. pseudomallei, B. thailandensis* was used to determine the specificity of the test condition. The isolates of *B. thailandensis were* obtained from the Faculty of Medicine, Khon Kean

University, and kept in 40% sterile glycerol at -80 °C before proceeding to determine the specificity of *orf2* and *orf11*. The bacterial stock was cultured on blood agar or MacConkey agar, and incubated at 37 °C for 48 h. Subsequently, the bacterial colonies were used for genomic DNA extraction by boiling method as described previously in Topic 3.2.2 and then the extracted DNA was used for detection of *orf2* and *orf11* by PCR technique as mentioned in Topic 3.2.3. The PCR products were electrophoresed through 2.0% agarose gel containing ViSafe Green Gel Stain (Vivantis, Malaysia) using 1X TBE buffer at 100 V for 45 min. Finally, the gel was visualized and photographed under ultraviolet light by Gel Doc (Vilber, France). The specificity of the designed gRNA in CRISPR/Cas12a system as well as the measurement signals of ssDNA-FQ reporter using FAM-BHQ1 were also investigated following the PCR technique. The procedures are previously described in Topic 3.2.5 and 3.2.6.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Detection of *orf2* and *orf11* of T3SS-1 of *B. pseudomallei* by polymerase chain reaction (PCR) amplification

The target sites of the present study were the open reading frames: *orf2* and *orf11* from T3SS-1 gene cluster that were the specific markers of *B. pseudomallei* distinct from another closely related species, *B. thailandensis* and *B. mallei* (Chantratita et al., 2008; Novak et al., 2006; Thibault et al., 2004). The genomic DNA of the wild type *B. pseudomallei* strain K96243 and isolates of *B. pseudomallei* from clinical samples were extracted by boiling method according to Armed and coworker, 2017. The extraction was performed with precipitation and without precipitation by absolute ethanol, followed by detection of *orf2* and *orf11* using the PCR technique. The PCR products of *orf2* and *orf11* were detected as 250 bp and 335 bp, respectively using 2% agarose gel electrophoresis. In this study, the genomic DNA of *B. pseudomallei*, Gram-negative bacteria, was extracted using a modified boiling method for rapid extraction, specifically for the purposes of PCR and CRISPR/Cas12a analysis. In addition, alcohol precipitation is commonly used for concentrating, desalting and recovering nucleic acids which precipitation is mediated by the addition of ethanol.

In the present study, both the wild type *B. pseudomallei* strain K96243 and clinical isolates of *B. pseudomallei* exhibited identical PCR products of *orf2* and *orf11* which were detected at 250 bp and 335 bp, respectively as shown in the Figure 4.1, 4.2 and 4.3. The results suggested that the primers designed for *orf2* and *orf11* in this study could be useful for investigation *B. pseudomallei* strain found in clinical samples. Furthermore, the results of this study demonstrated that DNA extracted by boiling method without ethanol precipitation appeared as a clear single band in the agarose gel similar to DNA extracted by boiling method with ethanol precipitation and DNA

extracted by boiling method with ethanol precipitation and incubated overnight at -20 ℃, which indicates that DNA was not degraded and provided the sufficient DNA yield. Hence, the boiling method offers a rapid, easy and cost-effective approach for high-yield DNA isolation from gram-negative bacteria. Additionally, this method circumvent the need for toxic chemicals.

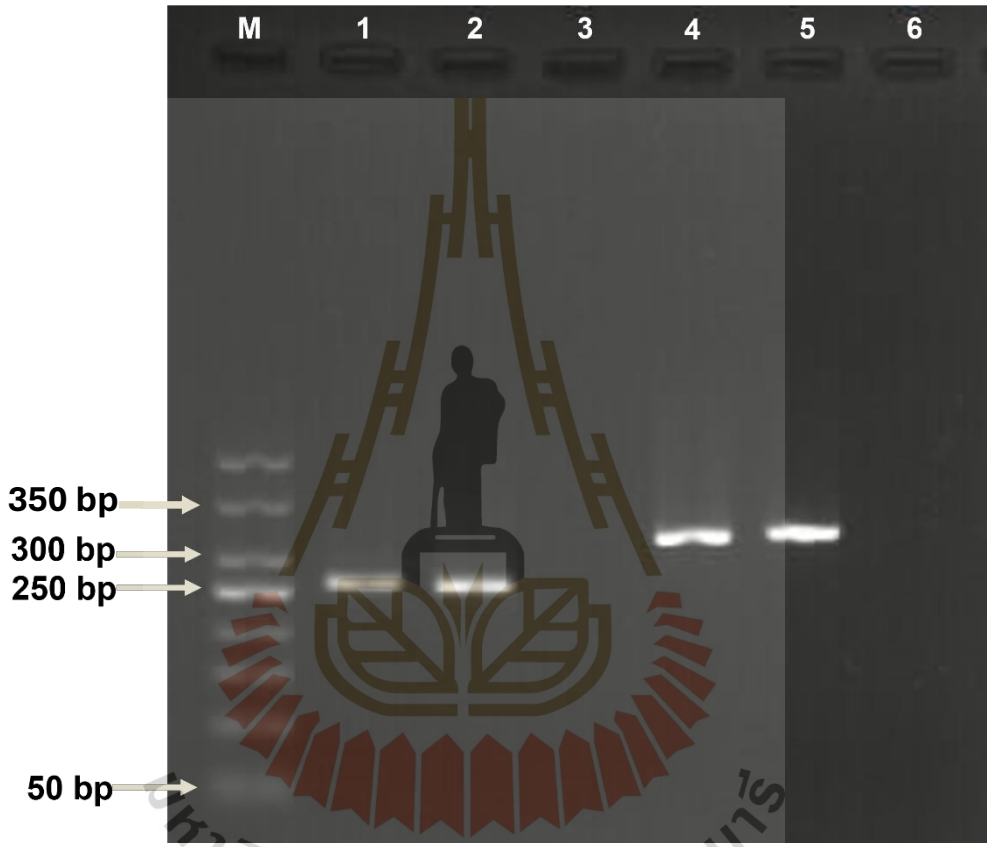

Figure 4.1 The PCR products of *orf2* and *orf11* from T3SS-1 of the wild type *B. pseudomallei* strain K96243 exhibited 250 bp and 335 bp, respectively using 2% agarose gel electrophoresis. Lane 1: PCR products of *orf2* which DNA extracted by boiling method with ethanol precipitation, Lane 2: PCR products of *orf2* which DNA extracted by boiling method without ethanol precipitation, Lane 3: negative control for *orf2*, Lane 4: PCR products of *orf11* DNA extracted by boiling method with ethanol precipitation, Lane 5: PCR products of *orf11* DNA extracted by boiling method with ethanol precipitation, Lane 6: negative control for *orf11*, Lane M:50-bp DNA ladder.

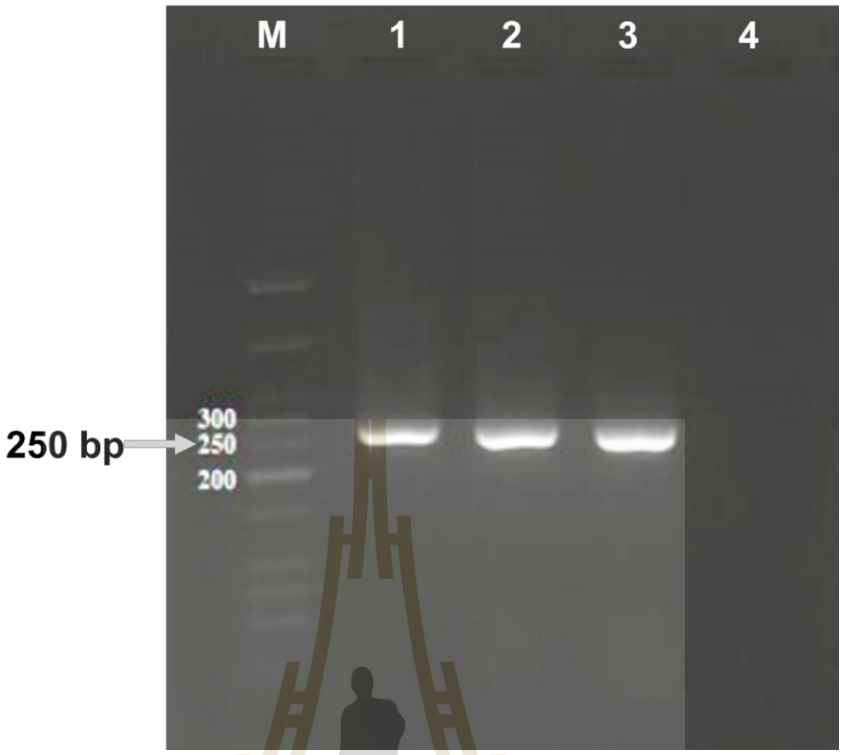

Figure 4.2 The PCR products of *orf2* from T3SS-1 of clinical isolate *B. pseudomallei* exhibited 250 bp using 2% agarose gel electrophoresis. Lane 1: DNA extracted by boiling method with ethanol precipitation, Lane 2: DNA extracted by boiling method without ethanol precipitation, Lane 3: DNA extracted by boiling method with ethanol precipitation and incubated overnight at -20 ℃, Lane 4: negative control Lane M: 25-bp DNA ladder.

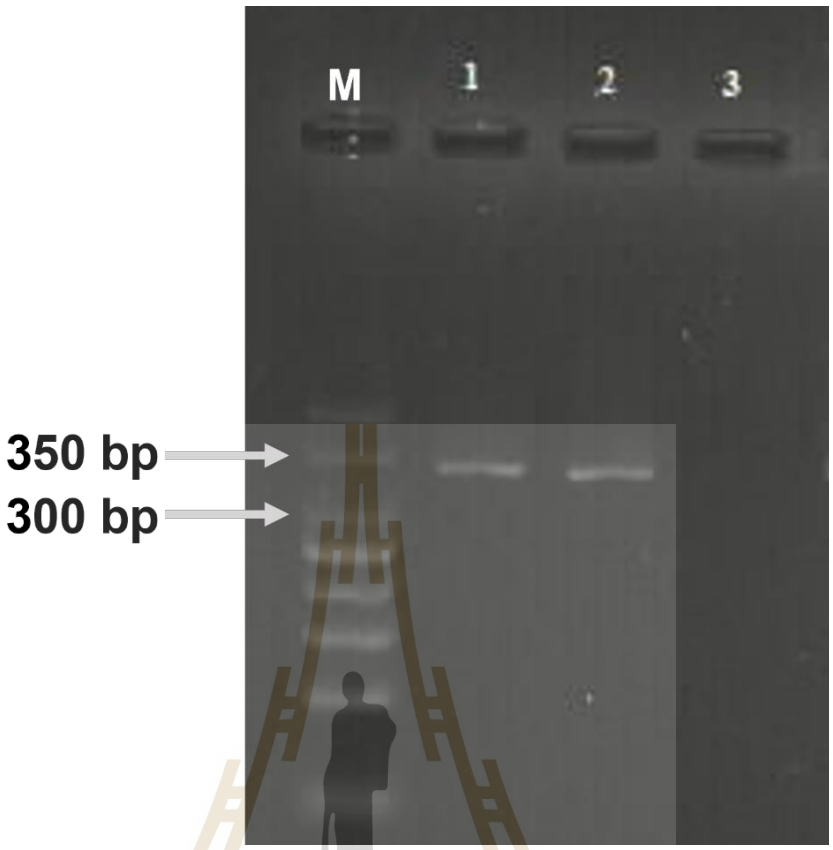

Figure 4.3 The PCR products of *orf11* from T3SS-1 of clinical isolate *B. pseudomallei* exhibited 335 bp using 2% agarose gel electrophoresis. Lane 1: DNA extracted by boiling method with ethanol precipitation, Lane 2: DNA extracted by boiling method with ethanol precipitation, Lane 3: negative control, Lane M: 50-bp DNA ladder.

4.2 Determination of the specificity of selected target site *orf2* and *orf11* from T3SS-1 *B. pseudomallei* with another Gram-negative bacilli

The soil saprophyte *B. thailandensis* is non-pathogenic for humans and animals and present abundant in the soils and standing waters of endemic areas. This species is closely related to *B. pseudomallei* and *B. mallei* as its genome encodes numerous homologs of virulence factors from these pathogenic species (Broek and Stevens, 2017; Thibault et al., 2004). In addition, *B. thailandensis* displays phenotypic characteristics that make it appear similar to *B. pseudomallei* by routine diagnosis tests. The *B. pseudomallei* genome encodes three T3SS which are referred to as T3SS-1, T3SS-2

and T3SS-3. The genomes of *B. pseudomallei* consists of two circular chromosomes., with all three T3SS residing on chromosome 2. T3SS-2 and T3SS-3 are present in the genomes of *B. mallei* and *B. thailandensis*, whereas T3SS-1 is absent from both (Broek and Stevens, 2017; Chantratita et al., 2008; Novak et al., 2006; Thibault et al., 2004). In the previous studies also reported that a 548-bp region of T3SS-1 of *B. pseudomallei* encompassing part of *orf2* was found to be present in *B. pseudomallei* and but not in related *B. mallei* or *B. thailandensis* (Novak et al., 2006). Furthermore, Thibault and co-worker also reported that *orf11* of T3SS-1 is a specific markers of *B. pseudomallei* distinct from other closely related species, *B. thailandensis* and *B. mallei*. Therefore, in this study the isolates of *B. thailandensis* obtained from Melioidosis Center, Faculty of Medicine, Khon Kaen University were used to determine the specificity of *orf2* and *orf11* from T3SS-1 of *B. pseudomallei* using PCR technique. The result revealed that none of the DNA fragments was observed when amplifying *orf11* from *B. thailandensis* by PCR technique as shown in Figure 4.5. Although a slightly intense DNA fragment of *orf2* was observed on the agarose gel, it exhibited a different fragment length from that of *B. pseudomallei* (250 bp), which was used as a positive control as shown in Figure 4.4. The result of *orf11* by PCR technique is consistent with the previous study indicated that *orf11* is a specific marker for *B. pseudomallei* (Thibault et al., 2004) and can be the specific target site for molecular diagnosis such as CRISPR/Cas12a. The finding of *orf2* fragment of *B. thailandensis* on the agarose gel should be improved by sequencing. However, the primers designed of *orf 2* in this study was able to detected *B. pseudomallei* at the DNA fragment length of 250 bp. Subsequently, the PCR products of *orf2* from *B. thailandensis* were tested in CRISPR/Cas12a system to determine the specificity of the designed gRNA used in this study.

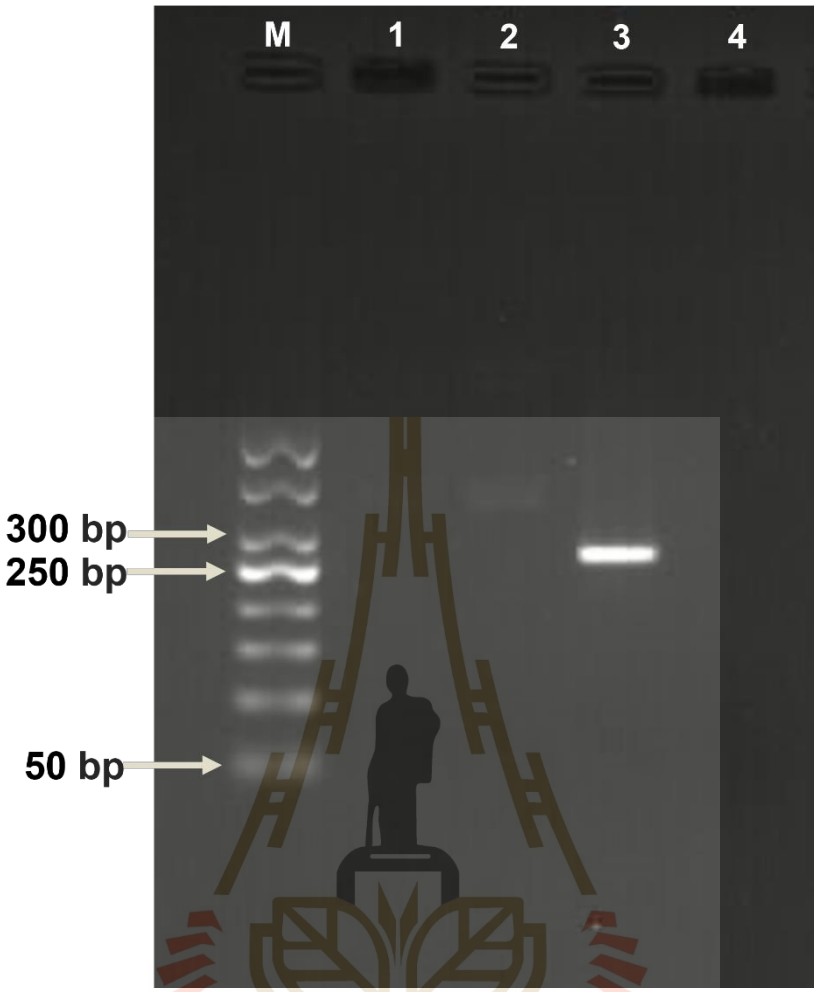

Figure 4.4 The PCR result of amplifying *orf2* from *B. thailandensis* was analyzed by 2% agarose gel electrophoresis to determine the specificity of *orf2* from T3SS-1 of *B. pseudomallei.* Lane 1: extracted DNA of *B. thailandensis* by boiling method with ethanol precipitation, Lane 2: extracted DNA of *B. thailandensis* by boiling method without ethanol precipitation, Lane 3: DNA of *B. pseudomallei* as a positive control (250 bp), Lane 4: negative control Lane M: 50-bp DNA ladder.

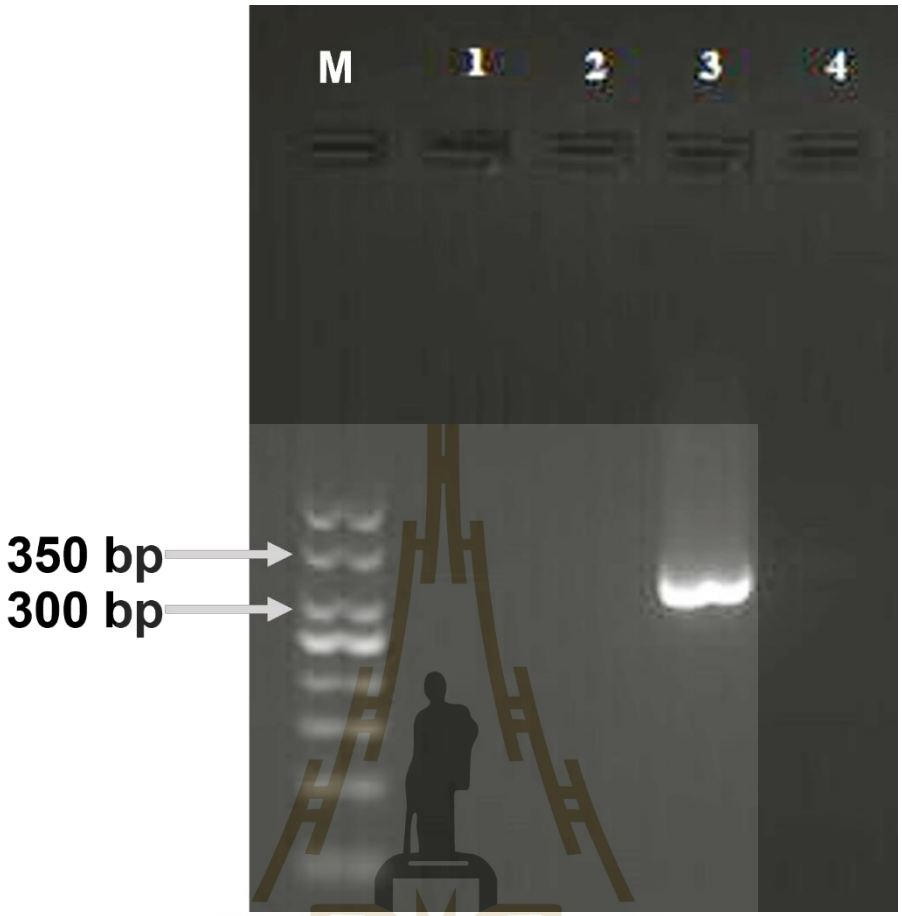

Figure 4.5 The PCR result of amplifying *orf11* from *B. thailandensis* was analyzed by 2% agarose gel electrophoresis to determine the specificity of *orf11* from T3SS-1 of *B. pseudomallei*. Lane 1: extracted DNA of *B. thailandensis* by boiling method with ethanol precipitation, Lane 2: extracted DNA of *B. thailandensis* by boiling method without ethanol precipitation, Lane 3: DNA of *B. pseudomallei* as a positive control (335 bp), Lane 4: negative control Lane M: 50-bp DNA ladder.

4.3 *In vitro* digestion activity of Lba Cas12a (Cpf1) on *B. pseudomallei* DNA target

The specificity of the designed gRNA to *B. pseudomallei* DNA target was tested using the PCR product of *orf2* and *orf11* from T3SS-1 of *B. pseudomallei* containing the target sequence. The designed gRNA of this study binding to target sequences of *orf2* and *orf11* were illustrated in Figure 4.6 and Figure 4.7, respectively. According to

previous studies, Cas12a quickly recognized and cleaved the DNA target site under the guidance of its specific gRNA (Moa et al., 2022). In our study, the *in vitro* digestion activity of RNA-guided enzyme Cas12a or CRISPR/Cas12a on target sites were investigated by 2.5% agarose gel electrophoresis. The results showed that the DNA fragments of *orf2* from both the wild type *B. pseudomallei* strain K96243 and the clinical isolate *B. pseudomallei* exhibited a larger size (350 bp) than its PCR product (250 bp) which were used as a control after adding gRNA and Cas12a into the reaction. Additionally, a smaller fragment of more than 50 bp was also observed (Figure 4.8 and Figure 4.9). Conversely, the DNA targets of *orf11* from both the wild type *B. pseudomallei* strain K96243 and the clinical isolate *B. pseudomallei* were cleaved into fragments ranging between 150 bp and 200 bp which were smaller than PCR product used as control (335 bp), as shown in Figure 4.8 and Figure 4.10. In theory, if the digestion occurs in the reaction involving the gRNA and Cas12a enzyme, it should have generated small sizes of the DNA fragments. However, in our study the *orf2* reaction showed 3 distinct bands, one of which showed a larger size of fragment band. This could be explained by the presence of tightly bound proteins (endonuclease enzyme) and nucleic acid (DNA) which form stable complexes during gel electrophoresis. The presence of complexes can cause slow-moving bands of DNA. This finding might further explain the efficiency of the gRNA of *orf2* and suggest that the gRNA may not be able to completely induce the digestion activity by Cas12a. Therefore, it might not be the best selected gRNA for the development of a diagnostic tool for a test with good sensitivity.

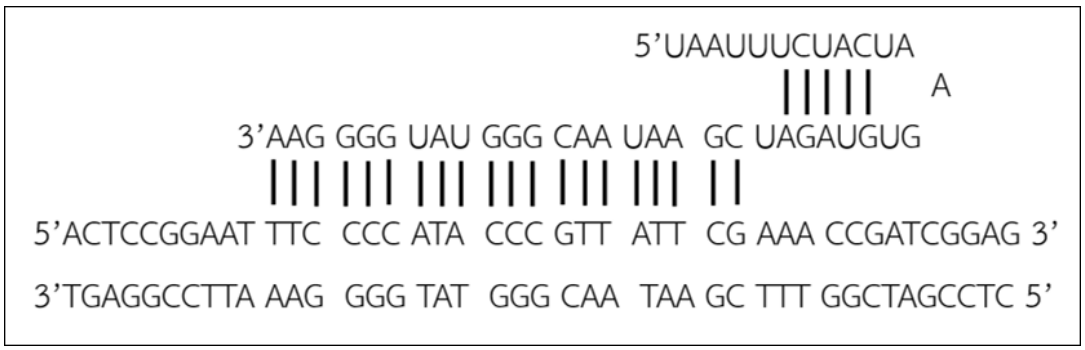

Figure 4.6 The gRNA binding to target sequences of *orf2* from T3SS-1 of *B. pseudomallei*.

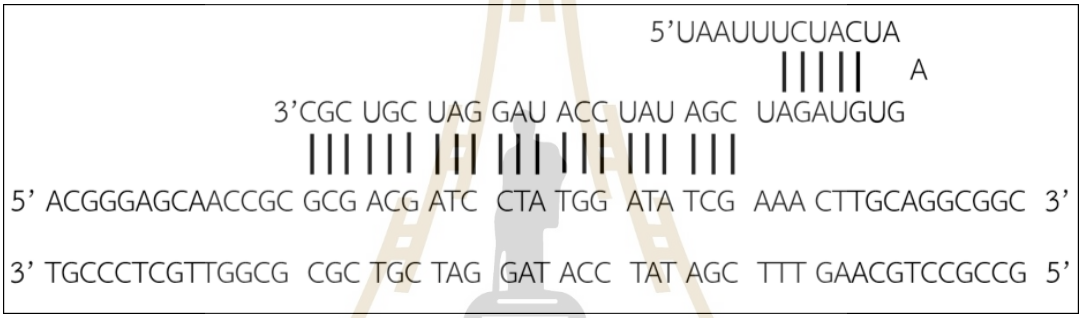

Figure 4.7 The gRNA binding to target sequences of *orf11* from T3SS-1 of *B. pseudomallei*.

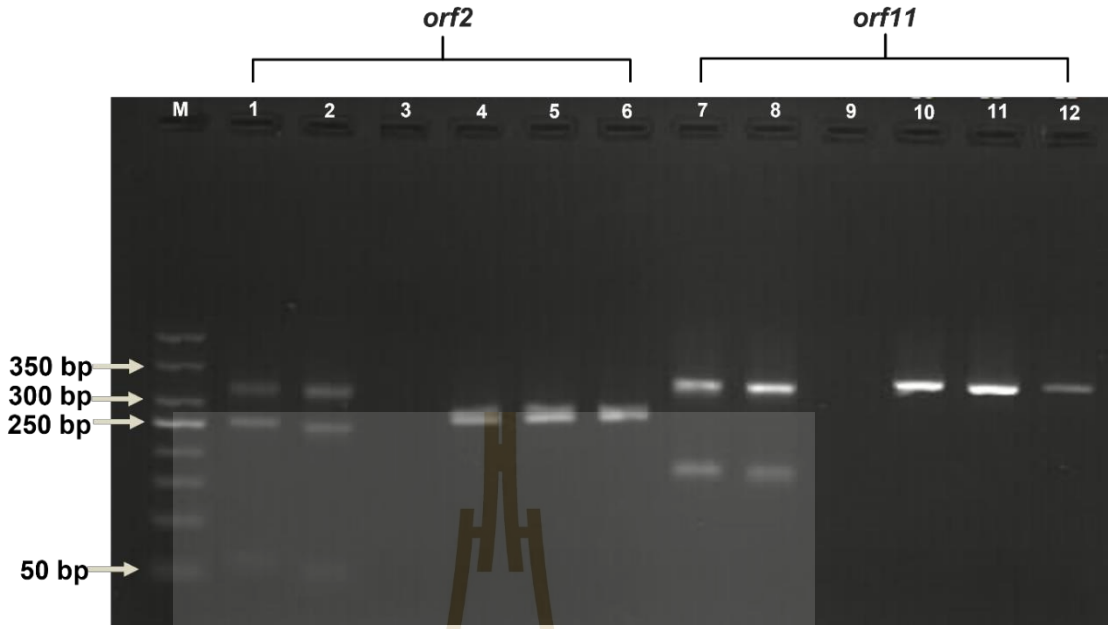

Figure 4.8 *In vitro* digestion activity of RNA-guided enzyme Cas12a or CRISPR/Cas12a on target sites of *orf2* and *orf11* from T3SS-1 of the wild type *B. pseudomallei* strain K96243 were investigated by electrophoresis with 2.5% agarose gel. Lane 1: DNA fragments of *orf2* exhibited different fragment sizes of 250, 350 and >50 bp after adding RNA-guided enzyme Cas12a (genomic DNA was extracted by boiling method with precipitation), Lane 2: DNA fragments exhibited different fragment sizes of 250, 350 and >50 bp after adding RNA-guided enzyme Cas12a (genomic DNA was extracted by boiling method without precipitation), Lane 3: gRNA and Cas12a without PCR product, Lane 4: PCR product and Cas12a except gRNA (genomic DNA was extracted by boiling method with precipitation), Lane 5: PCR product and Cas12a except gRNA (genomic DNA was extracted by boiling method without precipitation), Lane 6: PCR product, Lane 7: DNA fragments of *orf11* exhibited smaller size (>150 bp) than PCR product (335 bp) after adding RNA-guided enzyme Cas12a (genomic DNA was extracted by boiling method with precipitation), Lane 8: DNA fragments of *orf11* exhibited smaller size (>150 bp) than PCR product (335 bp) after adding RNA-guided enzyme Cas12a (genomic DNA was extracted by boiling method without precipitation), Lane 9: gRNA and Cas12a without PCR product, Lane 10: PCR product and Cas12a except gRNA (genomic DNA was extracted by boiling method with precipitation), Lane 11: PCR product and Cas12a

except gRNA (genomic DNA was extracted by boiling method without precipitation), Lane 12: PCR product, Lane M: 50-bp DNA ladder.

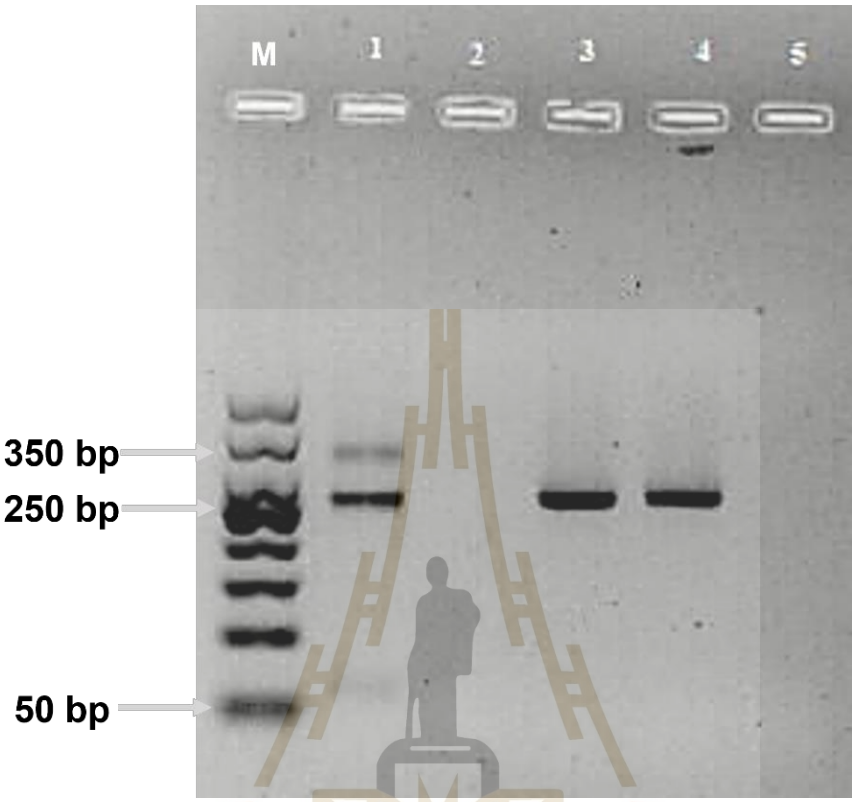

Figure 4.9 *In vitro* digestion activity of RNA-guided enzyme Cas12a or CRISPR/Cas12a on target sites of *orf2* from T3SS-1 of clinical isolate *B. pseudomallei* were investigated by electrophoresis with 2.5% agarose gel. Lane 1: DNA fragments exhibited different fragment sizes of 250, 350 and >50 bp after adding RNA-guided enzyme Cas12a, Lane 2: gRNA and Cas12a without PCR product, Lane 3: PCR product and Cas12a without gRNA, Lane 4: PCR product, Lane 5: negative control, Lane M: 50-bp DNA ladder.

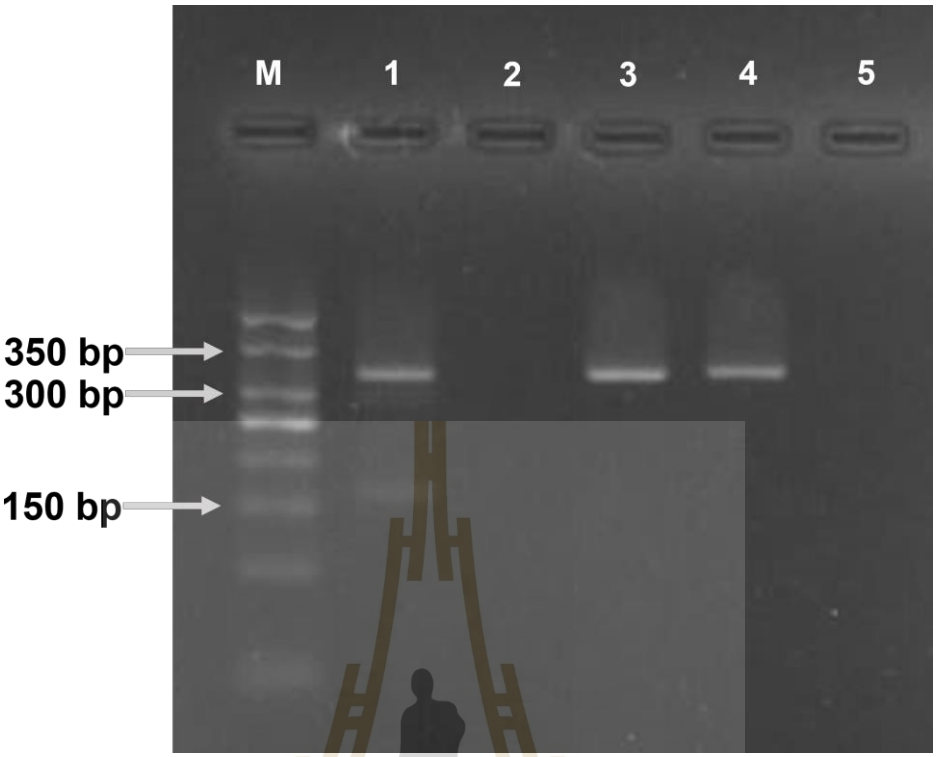

Figure 4.10 *In vitro* digestion activity of RNA-guided enzyme Cas12a or CRISPR/Cas12a on target sites of *orf11* from T3SS-1 of clinical isolate *B. pseudomallei* were investigated by electrophoresis with 2.5% agarose gel. Lane 1: DNA fragments exhibited smaller size (>150 bp) than PCR product (335 bp) after adding RNA-guided enzyme Cas12a, Lane 2: gRNA and Cas12a without PCR product, Lane 3: PCR product and Cas12a without gRNA, Lane 4: PCR product, Lane 5: negative control, Lane M: 50-bp DNA ladder.

4.4 Measurement the signal of single stranded DNA fluorophore-quencher (ssDNA-FQ reporter) of FAM-BHQ1 in CRISPR/Cas12a system

As Cas12a is considered to have a powerful non-specific cleavage activity to ssDNA after binding to the target DNA. Hence, this study further measured the digestion activity of CRISPR/Cas12a at the DNA target of *B. pseudomallei* by evaluation the signal of ssDNA-FQ reporter carrying fluorescein reporter molecule attached at 5′ end and black hole quencher1 on the 3′ end (FAM-BHQ1). The output fluorescence signal and its intensity are directly related to the presence and concentration of the activated

Cas12a in the reaction system (Xiong, 2020). So, the signal of fluorophore of the cleavage products was monitored every single min until 2 h compared with the fluorescence signal of control groups. The positive signal of fluorescence indicates that RNA-guided enzyme designed in this study specifically reacted with *B. pseudomallei* DNA target in CRISPR/Cas12a system as well as collateral cleavage nearby ssDNA-FQ reporters.

In our study, the target-activated CRISPR/Cas12a cleavage activity was verified based on signal amplification of ssDNA-FQ reporter. The result revealed that an increase of collateral cleavage activity of the FAM fluorophore from its quencher, leading to an increased generation of fluorescence signal, which was observed in both *orf2* and *orf11* of the wild type *B. pseudomallei* strain K96243 and *B. pseudomallei* isolated from clinical sample but not in *B. thailandensis* and control samples (Figure 4.11 and Figure 4.12). Although, the finding of slightly intense *orf2* fragment of *B. thailandensis* on the agarose gel by PCR, further analysis using the CRISPR/Cas12a system with ssDNA-FQ reporter demonstrated that the designed gRNA for *orf2* from T3SS-1 of *B. pseudomallei* of this study was specific for detecting *B. pseudomallei* (Figure 4.11). Furthermore, *B. thailandensis* was not detected using the PCR technique, indicating that *orf11* served as a specific marker for *B. pseudomallei*. Further analysis using the CRISPR/Cas12a system with an ssDNA-FQ reporter demonstrated that the designed gRNA targeting *orf11* from T3SS-1 was specific for *B. pseudomallei* detection (Figure 4.12). These indicated that both gRNA of *orf2* and *orf11* designed in this study could specifically detect *B. pseudomallei*, but not other pathogens.

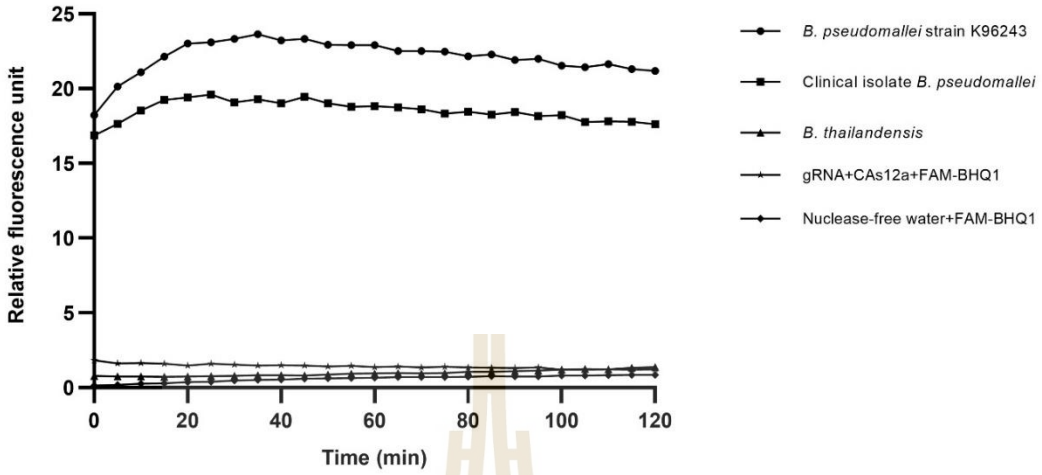

Figure 4.11 The signal of FAM fluorophore of cleavage products of *orf2* from T3SS-1 of *B. pseudomallei* comparing with the fluorescence signals of control groups.

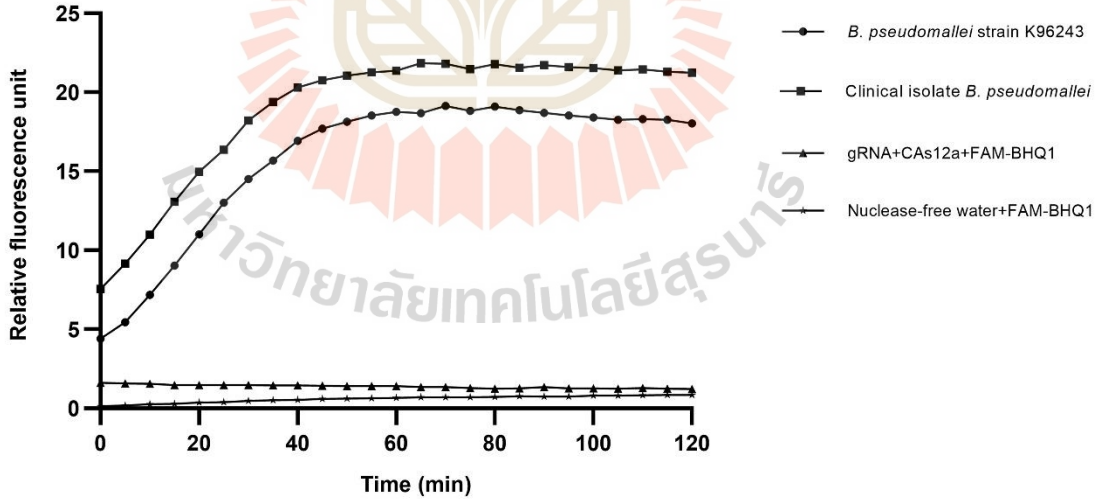

Figure 4.12 The signal of of FAM fluorophore of cleavage products of *orf11* from T3SS-1 of *B. pseudomallei* comparing with the fluorescence signals of control groups.

CHAPTER V
CONCLUSION

Burkholderia pseudomallei is the etiology agent of an infectious disease known as melioidosis. The disease has been in humans ranges from asymptomatic to focal infection and could be life-threatening by rapid fatal septicemia. The treatment of melioidosis requires drug of choices which are ceftazidime and co-trimoxazole. An early diagnosis of the disease could decrease the fatal rate of the patients. Clinical presentation and rapid detection of the microorganism are necessary for early diagnosis of melioidosis. The conventional culture of the microbe is time-consuming and requires a full set of biochemical tests. In addition, automatic machinery for identification of such bacteria are not available in small microbiology laboratories. Therefore, a simple and rapid detection with high specificity and sensitivity of the test is required for its early diagnosis. CRISPR-Cas12a technology is an attractive tool for infectious disease diagnostic applications. This technology involves two essential components: a guide RNA to match a desired target gene, and Cas12a as an endonuclease for digestion activity.

In this study, we developed a new methodology for a rapid detection of *B. pseudomallei* using CRISPR-Cas12a technology. The T3SS is an important virulence factor of *B. pseudomallei* which T3SS gene cluster encodes a toxin delivery mechanism allowing pathogenic bacteria to inject toxic substances into the cytoplasm of the host's cells. The *B. pseudomallei* genome encodes three T3SSs which are referred to as T3SS-1, T3SS-2 and T3SS-3. T3SS-1 is present in *B. pseudomallei*, but absent in both *B. mallei* and *B. thailandensis* which are closely related Gram-nagative bacteria. Therefore, the open reading frames: *orf2* and *orf11* from T3SS-1 gene cluster that were the specific markers of *B. pseudomallei* distinguishing from another closely related species, *B. thailandensis* and *B. mallei* were selected for gRNA design in this study. The DNA of *B. pseudomallei* was extracted from the colony suspension by different methods and the boiling method without DNA precipitation gave the quickest and simplest

methodology for DNA extraction. The newly designed gRNA was shown to be specific for *orf2* and generated digested DNA fragments with larger and smaller sizes whereas the newly designed gRNA specific for *orf11* generated smaller sizes of digested DNA fragments. This indicates that the two newly designed gRNA were specific for this bacterial pathogen illustrating the endonuclease activity, therefore, they could be applied for the rapid detection of *B. pseudomallei* in clinical specimens collected from melioidosis patients. To visualize the digestion activity, a ssDNA-FQ reporter should be used for simple readouts of the target-activated CRISPR/Cas12a cleavage activity. The output fluorescence signal and its intensity are directly related to the presence and concentration of the activated Cas12a in the reaction system. In our study, we observed an increase in collateral cleavage activity of the FAM fluorophore from its quencher, according to an increased generation of fluorescence signal, in the wild-type *B. pseudomallei* strain K96243 and *B. pseudomallei* isolated from clinical samples, but not in *B. thailandensis* or control samples. Although, the finding of slightly intense *orf2* fragment of *B. thailandensis* on the agarose gel by PCR was observed, further analysis using the CRISPR/Cas12a system with ssDNA-FQ reporter demonstrated that the designed gRNA for *orf2* from T3SS-1 of *B. pseudomallei* of this study was specific for detecting *B. pseudomallei* based on fluorescence signal amplification. Furthermore, the designed gRNA for *orf11* from T3SS-1 of *B. pseudomallei* also demonstrated the specificity for detecting *B. pseudomallei* because of no fragment was found on agarose gel by PCR and the increasing of fluorescence signal was observed. These indicated that both gRNA of *orf2* and *orf11* designed in this study could specifically detect *B. pseudomallei*, but not other pathogens. As the results, these newly designed gRNA can be used for the development of a rapid diagnostic tool for melioidosis. This could facilitate a clinical microbiological laboratory in small community hospitals to detect and interpret the infection from *B. pseudomallei* more efficiently. For the further study the sensitivity of gRNA of *orf2* and *orf11* for *B. pseudomallei* detection should be determined.

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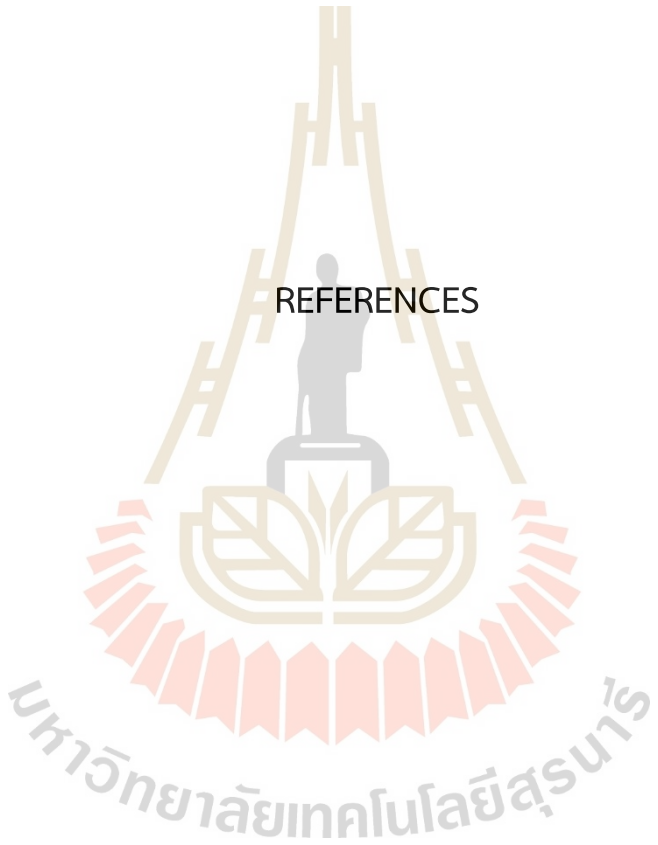

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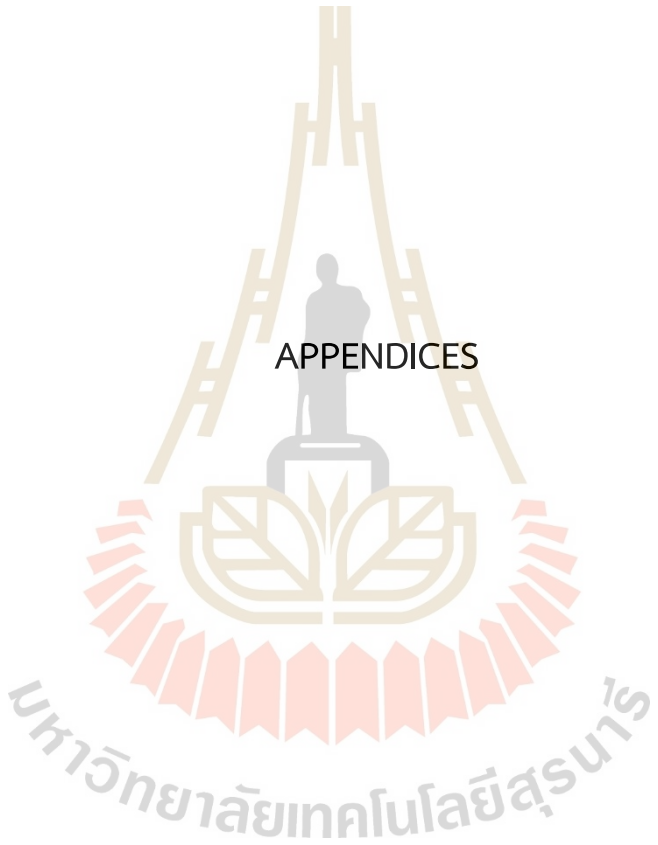
APPENDICES



APPENDIX A

SEQUENCE OF OPEN READING FRAMES 2 (*ORF2*) AND OPEN READING FRAMES 11 (*ORF11*) FROM T3SS-1 OF *B. PSEUDOMALLEI* K96243

ORF2

>AF074878.2: 25391-26344 *Burkholderia pseudomallei* putative type III secretion system-associated gene cluster, complete sequence

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ORF11

>AF074878.2:1064-4927 *Burkholderia pseudomallei* putative type III secretion system-associated gene cluster, complete sequence

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TTCGGACCGCCTCGAACTCAAATCACTTTGATGAATGGATGCACCGCTTGATCGAATAATCACA
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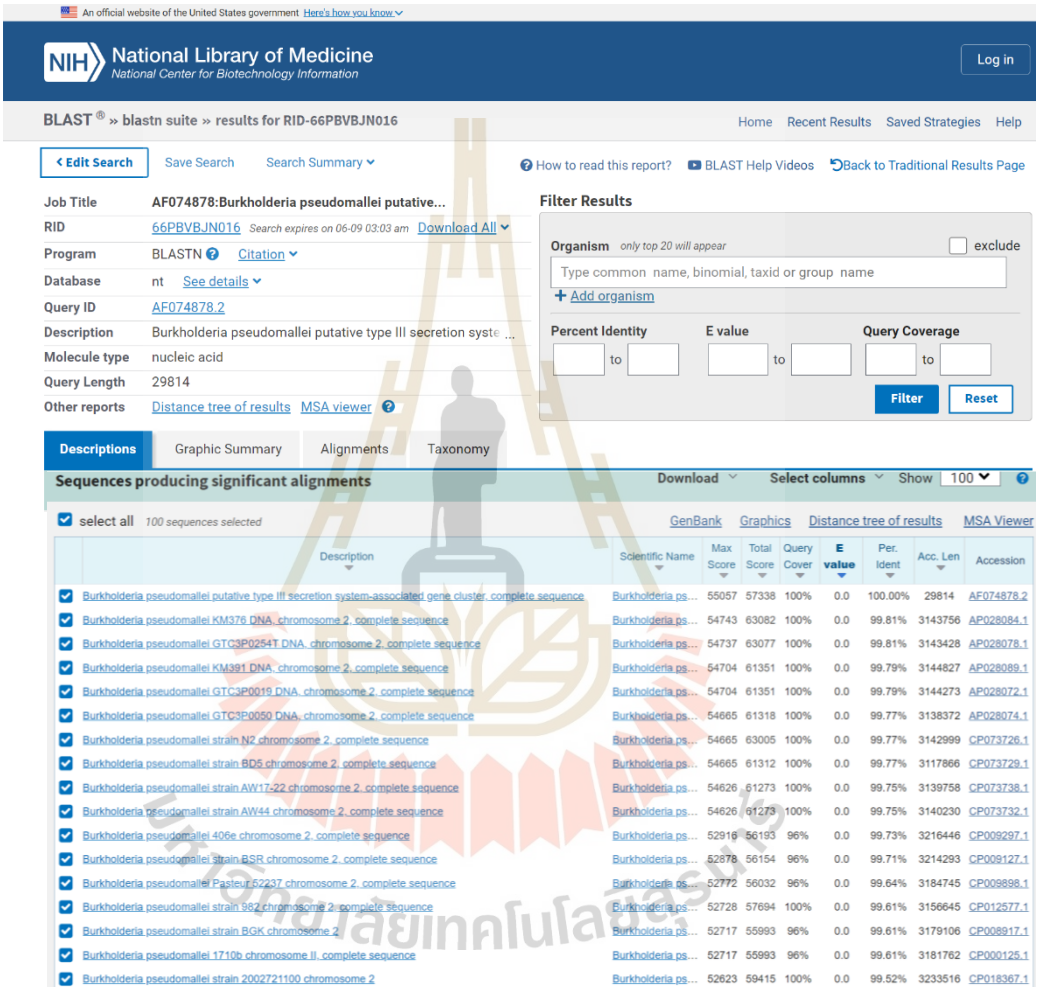
APPENDIX B

BLASTN RESULTS OF *B. PSEUDOMALLEI* K96243

AND PRIMERS BLAST OF ORF2 AND ORF11



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Job Title	AF074878:Burkholderia pseudomallei putative...
RID	66PBVBJN016 Search expires on 06-09 03:03 am Download All
Program	BLASTN Citation
Database	nt See details
Query ID	AF074878.2
Description	Burkholderia pseudomallei putative type III secretion syste ...
Molecule type	nucleic acid
Query Length	29814
Other reports	Distance tree of results MSA viewer

Filter Results

Organism only top 20 will appear exclude

Type common name, binomial, taxid or group name

+ Add organism

Percent Identity to | E value to | Query Coverage to

Filter Reset

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments

Download Select columns Show 100

select all 100 sequences selected

GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Burkholderia pseudomallei putative type III secretion system-associated gene cluster, complete sequence	Burkholderia ps...	55057	57338	100%	0.0	100.00%	29814	AF074878.2
Burkholderia pseudomallei KM376 DNA, chromosome 2, complete sequence	Burkholderia ps...	54743	63082	100%	0.0	99.81%	3143756	AP028084.1
Burkholderia pseudomallei GTC3P0254T DNA, chromosome 2, complete sequence	Burkholderia ps...	54737	63077	100%	0.0	99.81%	3143428	AP028078.1
Burkholderia pseudomallei KM391 DNA, chromosome 2, complete sequence	Burkholderia ps...	54704	61351	100%	0.0	99.79%	3144827	AP028089.1
Burkholderia pseudomallei GTC3P0019 DNA, chromosome 2, complete sequence	Burkholderia ps...	54704	61351	100%	0.0	99.79%	3144273	AP028072.1
Burkholderia pseudomallei GTC3P0050 DNA, chromosome 2, complete sequence	Burkholderia ps...	54665	61318	100%	0.0	99.77%	3138372	AP028074.1
Burkholderia pseudomallei strain N2 chromosome 2, complete sequence	Burkholderia ps...	54665	63005	100%	0.0	99.77%	3142999	CP073726.1
Burkholderia pseudomallei strain BD5 chromosome 2, complete sequence	Burkholderia ps...	54665	61312	100%	0.0	99.77%	3117866	CP073729.1
Burkholderia pseudomallei strain AW17-22 chromosome 2, complete sequence	Burkholderia ps...	54626	61273	100%	0.0	99.75%	3139758	CP073738.1
Burkholderia pseudomallei strain AW44 chromosome 2, complete sequence	Burkholderia ps...	54626	61273	100%	0.0	99.75%	3140230	CP073732.1
Burkholderia pseudomallei 406e chromosome 2, complete sequence	Burkholderia ps...	52916	56193	96%	0.0	99.73%	3216446	CP009297.1
Burkholderia pseudomallei strain BSR chromosome 2, complete sequence	Burkholderia ps...	52878	56154	96%	0.0	99.71%	3214293	CP009127.1
Burkholderia pseudomallei Pasteur 52237 chromosome 2, complete sequence	Burkholderia ps...	52772	56032	96%	0.0	99.64%	3184745	CP009898.1
Burkholderia pseudomallei strain 982 chromosome 2, complete sequence	Burkholderia ps...	52728	57694	100%	0.0	99.61%	3156645	CP012577.1
Burkholderia pseudomallei strain BGK chromosome 2	Burkholderia ps...	52717	55993	96%	0.0	99.61%	3179106	CP008917.1
Burkholderia pseudomallei 1710b chromosome II, complete sequence	Burkholderia ps...	52717	55993	96%	0.0	99.61%	3181762	CP000125.1
Burkholderia pseudomallei strain 2002721100 chromosome 2	Burkholderia ps...	52623	59415	100%	0.0	99.52%	3233516	CP018367.1

ORF2 primers

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CTCACTTCGAAGCCGAACC	Plus	19	25719	25737	58.55	57.89	8.00	0.00
Reverse primer	AGTCCGAACATCTCGCTCTC	Minus	20	25968	25949	59.26	55.00	4.00	0.00
Product length	250								

Products on intended targets

>AF074878.2 Burkholderia pseudomallei putative type III secretion system-associated gene cluster, complete sequence

```
product length = 250
Forward primer  1      CTCACTTCGAAGCCGAACC  19
Template        25719  ..................  25737

Reverse primer  1      AGTCCGAACATCTCGCTCTC  20
Template        25968  ..................  25949
```

ORF11 primers

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AAGCGTAGGCGAAACACTGA	Plus	20	2426	2445	59.97	50.00	4.00	1.00
Reverse primer	ACGATGCGGTCAAAAGGAG	Minus	19	2760	2742	58.16	52.63	2.00	0.00
Product length	335								

Products on intended targets

>AF074878.2 Burkholderia pseudomallei putative type III secretion system-associated gene cluster, complete sequence

```
product length = 335
Forward primer  1     AAGCGTAGGCGAAACACTGA  20
Template        2426  ..................  2445

Reverse primer  1     ACGATGCGGTCAAAAGGAG  19
Template        2760  ..................  2742
```

APPENDIX C

EQUIPMENTS AND INSTRUMENTS

Name	Source
Autoclave	Hirayama, Japan
Autopipettes (1, 20,100, 1000 ul.)	Satorius, Finland
Balance	Denver instrument, Germany
Electrophoresed	ELITE 300 plus,Wealtec corp.
Flask (250 ml)	Pirex, USA
Centrifuge	HERMLE, Germany
Class II Biohazard Safety Cabinet	Thermo scientific, USA
Incubator	SHEL LAB, Sheldon mfg. Inc.
96-well plate	Thermo scientific Nunc, Denmark
Heat block	Hangzhou Miu instruments
Fluorescence plate reader	Thermo varioscanlux
Mixture	Scientific industries
Nanodrop	Thermo Fisher Scientific, DE,USA
Tips(10,200,1000ul)	Thermo scientific, Mexico
Thermocycler	BIO-RAD T100,Singapore

CURRICULUM VITAE

Name	Mrs. Pimpaka Rongkratok
Date of Birth	February 27, 1975
Place of Birth	Nakhon Ratchasima, Thailand
Education:	
1994 - 1998	Bachelor of Science (Medical technology), Khon Kaen University, Khon Kaen, Thailand
2018 - Present	Master of Science student (Biomedical sciences), School of Preclinical Sciences, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand
Work Experience :	
1998 – Present	Medical Technologist, Nonsung Hospital, Nakhon Ratchasima, Thailand