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 ℃ for 48 h. The bacteria were transferred to a sterile cryotube containing 60% nutrient broth and 40% sterile glycerol and then stored at -80 ℃ 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 ℃, 15 psi for 15 min and then cooled it in the water bath until the temperature down to 50-55 ℃ 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 ℃ 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 ℃, 15 psi for 15 min and then cooled to 45-50 ℃ 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 ℃).

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 ℃, 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 ℃.

3.1.3 Chemicals and Reagents

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

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.