CHAPTER III
RESEARCH METHODOLOGY

1. Materials

1.1 Chemicals

All chemicals used in experiments were analytical grade. Names and sources of chemical were listed below.

Chemicals	Sources
Agarose	Sigma
Absolute ethanol (C_2H_5OH)	Merck
Dipotassium hydrogen phosphate (K_2HPO_4)	Fluka
Dipotassium hydrogen phosphate dihydrate($K_2HPO_4.2H_2O$)	Merck
Ethylene diamine tetrachloroaceic acid [EDTA ($C_{10}H_{14}N_2Na_2O_8.2H_2O$)	Merck
Magnesium sulphate ($MgSO_4.7H_2O$)	May&Baker
Potassium chloride (KCL)	Carlo Erba
Potassium dihydrogen phosphate (KH_2PO_4)	Merch
Sodium ammonium hydrogen phosphate tetrahydrate ($NaNH_4HPO_4.7H_2O$)	Fluka
Sodium chloride (NaCl)	Carlo Erba
Sodium hydrogen carbonate ($NaHCO_3$)	Merck
Sodium hydrogen phosphate ($NaHPO_4$)	Merck

1.2 Instruments

Instruments	Sources
Autopipette 0.5-10 µl	JENCONS
Autopipette 5-50 µl	JENCONS
Autopipette 200-1000 µl	JENCONS
DNA thermal cycler (2400; Perkin-Elmer)	Norwalk, CT

Instruments	Sources
Incubator	Mammert
Incubation shaker	Innova™
ELISA reader	Sunrise
Heat box	Boekel
Hot air oven	WT binder
Microcentrifuge	Hettich
Mini VE gel caster	Hoefer
pH meter	Fish Science
Refrigerator (-70°C)	Kelvinator
Refrigerator (-20°C)	Sanyo
Refrigerated centrifuged, model J2-21	Beckman
Spectrophotometer	Amersham
Vortex mixer	Velp Science
Water bath	Hetotherm®

1.3 Media

Media	Sources
Agar	Pronadisa
Tryptic soy broth	BD
Bovine serum albumin	Bio West

1.4 Reagent kits

Reagent kits	Sources
Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP	Dako
Substrate	R&D system
Mouse IFN-γ set	R&D system
wcbG_primer	Invitrogen™

1.5 Bacterial strains

The virulent strain of *B. pseudomallei* (A2) was used in this study. It was isolated from blood of Thai patient with septicemic melioidosis. This isolate

exhibited typical LPS type as done by SDS-PAGE (Anuntagool et al., 2000). The 50% lethal dose (LD_{50}) of this strain is 19 (Taweechaisupapong et al., 2005).

1.6 Animals

BALB/c mice, 6 to 8 weeks-old (National Laboratory Animal Center, Mahidol University, Salaya, Nakornpatom, Thailand) were used because this inbred strain has been shown to be highly susceptible to *B. pseudomallei* infection (Leakey et al., 1998; Ulett et al., 2000). Animals were maintained under strictly hygienic conventional conditions at the Laboratory Animals Breeding Unit, Faculty of Medicine, Khon Kaen University. All experimental procedures performed on the animals were approved by the Animal Ethics Research Committee of the Faculty of Medicine, Khon Kaen University.

2. Study designs

The proposed plan of this study was carried out as followed:

2.1 To study the kinetic growth of *B. pseudomallei* and specific antibody responses in acute infected BALB/c mice (Fig. 6).

2.1.1 Forty BALB/c mice were infected with $12LD_{50}$ (230 CFU) of *B. pseudomallei* by intraperitoneal (IP) injection (for acute infection). The mice were divided into 5 groups. Groups 1-5 were infected with *B. pseudomallei* and their blood and organs were collected after 12, 24, 48, 60 and 72 h respectively. Phosphate buffered saline (PBS) injected group (six mice) were used as control

2.1.2 At various time points after infection (12 , 24 , 48 , 60 and 72 h), the heparinized blood was collected aseptically from the remaining infected mice then lungs, livers and spleens were removed. The samples were used to determine the kinetics of specific antibody in plasma by ELISA and the present of *B. pseudomallei* in blood and various organs by plate count technique and polymerase chain reaction (PCR).

2.2 To study the kinetic growth of *B. pseudomallei* and specific antibody responses in chronic *B. pseudomallei* infected BALB/c mice (Fig. 7).

2.2.1 Fifteen BALB/c mice were infected with $0.3LD_{50}$ (6 CFU) of *B. pseudomallei* by intraperitoneal injection. Another five mice were injected with PBS for control group.

2.2.2 Heparinized blood were collected aseptically from infected mice at 1st, 3rd, 5th, 7th, 14th, 21st and 28th days post-infection. The blood were used to determine the kinetic of specific antibody responses in plasma by ELISA and *B. pseudomallei* in blood by plate count technique and polymerase chain reaction (PCR).

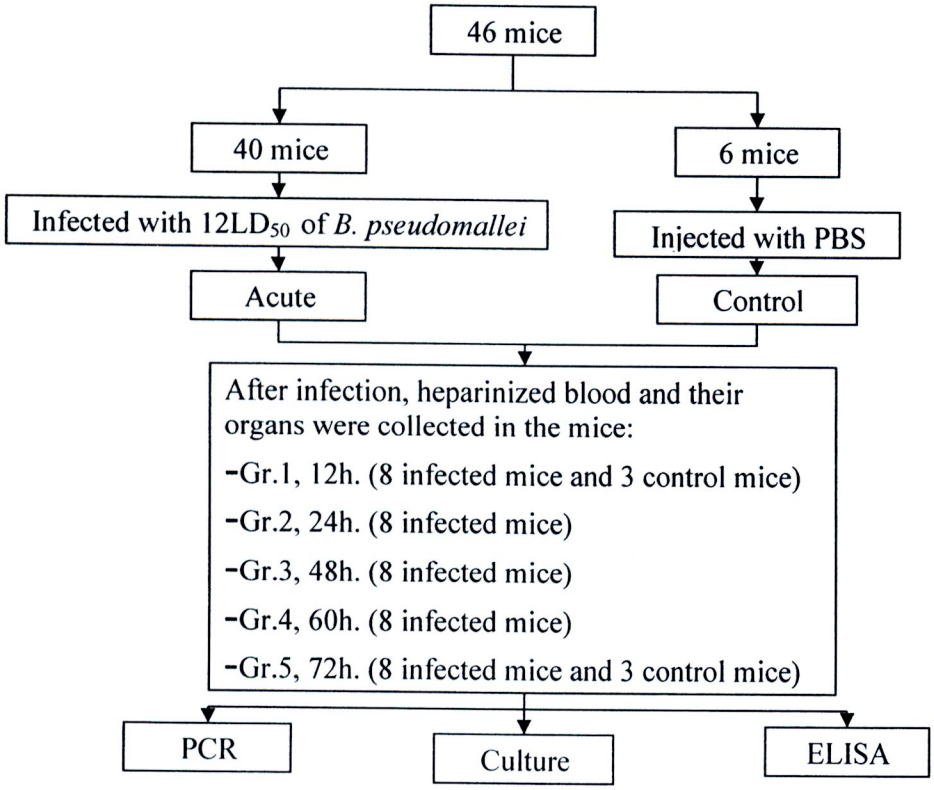

Figure 6 The study design of kinetic growth of *B. pseudomallei* and specific antibody responses in blood and organs of acute infected BALB/c mice

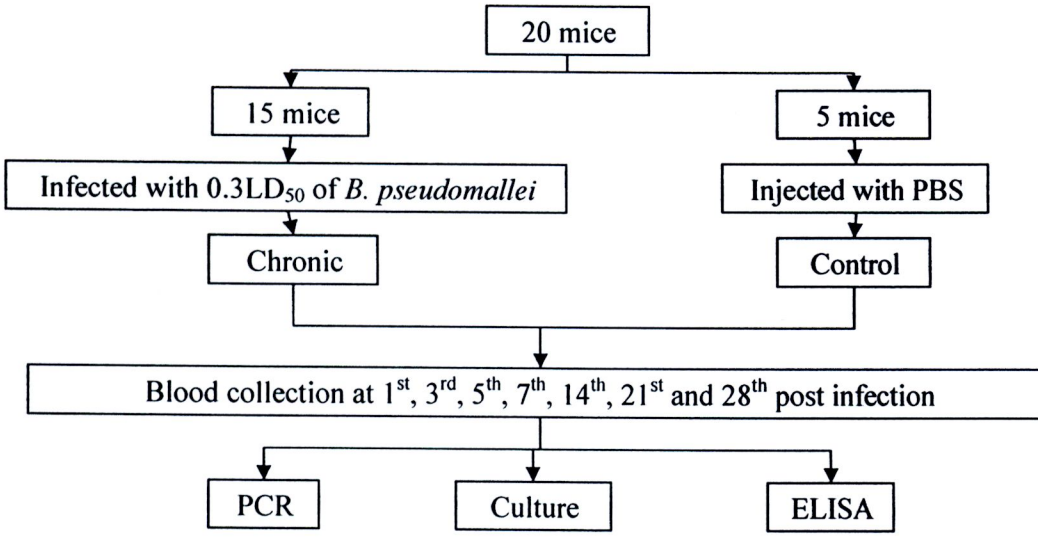

Figure 7 The study design of kinetic growth of *B. pseudomallei* and specific antibody responses in blood of chronic infected BALB/c mice

3. Methods

3.1 Bacterial preparation

The virulent strain of *B. pseudomallei* (A2) was grown on Ashdown agar at 37° C for 48 h. The single colony was picked and grown aerobically in 3 ml of tryptic soy broth (200 rpm) at 37° C overnight. One ml of bacterial suspension was added to 50 ml of TSB and further incubated (200 rpm) at 37° C for 2.5 h to reach the mid log phase of growth.

3.2 Mice infection

For each infection experiment, the bacterial cells were diluted in PBS to obtain the appropriate concentration ($12LD_{50}$ and $0.3LD_{50}$ for induce acute and chronic infections respectively). The actual number of bacteria was determined by plating 0.1 ml of undiluted and serial 1:10 dilution on Ashdown's medium and counting CFU after 48 h. The 100 µl of each bacterial suspension were injected to BALB/c mice by intraperitoneal (IP) injection and PBS was used for the control group. In acute infection, the blood sample was collected from each anaesthetized mice via retroorbital puncture with a sterile heparinized capillary tube, then their lungs, livers and spleens were aseptically excised at 12, 24, 48, 60 and 72 h. after infection. For

chronic infected mice, their heparinized blood were collected with the same technique at 1st,3rd,5th,7th,14th 21st and 28th days post infection. The viable cell count, PCR and antibody responses were determined as described in study design.

3.3 Determination of the kinetic of viable *B. pseudomallei* in blood and various organs by culture and PCR technique.

3.3.1 Determination of bacterial load in blood and organs of infected mice by culture

At various time post infection, the number of viable *B. pseudomallei* present in blood and various organs was determined. The lungs, livers and spleens were aseptically removed and homogenized in 1 or 3 ml (depending on organ size) of PBS by passed through a 70 μm-pore-size nylon sieve. A 0.1 ml of undiluted and 1:10 dilution of samples were plated on Ashdown's medium and the number of CFU was determined. The colonies on plates were counted after 2 days of incubation at 37°C and the bacteria were confirmed by slide agglutination test (Samosornsuk et al., 1999).

3.3.2 Preparation of blood samples and various organs for PCR

The samples were centrifuged (12000 rpm for 5 min). After taken the plasma, the red blood cells were lysed by the addition of 1 ml of sterile distilled water, vortexed and then centrifuged at 12000 rpm for 5 min for three times (complete hemoglobin removed). The pellet were washed twice with TE (10 mM Tris-HCl, 10 mM EDTA, pH 8.0), resuspended in 100 μl of TE then boiled for 10 min. Prior to PCR, the samples were centrifuged at 12000 rpm for 5 sec. and 5 μl of supernatant was used for amplification. Each sample, PCR was used primers for β-actin as an internal positive-control, that were performed as previously described (Ulett et al., 2000). In a negative control PCR was run distilled water).

3.3.3 Preparation of DNAs for sensitivity of *B. pseudomallei* detection (Miller et al., 1988)

Bacterial DNAs were extracted by the method of Miller *et al.*, with some modifications. The bacteria were grown overnight at 37°C in 1.5 ml LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract, 0.5% NaCl). The cells were harvested by centrifugation at 12000 rpm for 3 min. The cell pellet was resuspended in 500 μl of extraction buffer (25mM Tris-HCL,50mM glucose, 100 mM NaCl, 10mM EDTA, pH

8.0). The suspension was mixed with an equal volume of proteinase K solution (0.1 mg ml^{-1} proteinase K in 4% SDS solution). The mixture was incubated at 65 °C for 2 to 3 h or until digestion was complete and 1/3 volume of saturated NaCl (approximately 5 M) was added. The suspension was shaken vigorously and then centrifuged at 12000 rpm for 10 min. The supernatant was precipitated with 2 volume of absolute ethanol. After thoroughly mixing, the DNA was spooled out with a glass rod and wash with 70% alcohol, then centrifuged at 12000 rpm for 10 min before dissolved in TE (10 mM Tris-HCl, pH 8.0, 10 mM EDTA).

3.3.4 Preparation of *B. pseudomallei* viable cells for sensitivity of PCR (Rattanathongkom et al., 1997)

Ten-fold serial dilution of overnight cultures of *B. pseudomallei* was made in sterile distilled water ($10\text{-}10^5$) and viable counts were determined by spreading 100 µl of each dilution on Ashdown agar. The cultures were incubated at 37° C for 48 h. At the same time, 1 ml of each dilution was centrifuged (12000 rpm for 5 min). The pellet was washed twice with TE, resuspended in 100 µl of TE then boiled for 10 min. Prior to PCR, the suspensions were centrifuged at 12000 rpm for 5 second and 5 µl of the supernatant was used for amplification.

3.3.5 PCR amplification

The PCR amplification of *B. pseudomallei* DNA was performed using a DNA thermal cycler (2400; Perkin-Elmer, Norwalk, CT). Bacterial DNA and ten-fold serial dilutions of both *B. pseudomallei* total DNAs and *B. pseudomallei* viable cells were amplified for 40 cycles by using primers wcbG_for (5'- AACGAGTCGGTCA TTTCCCTGA-3') and wcbG_rev (5'-CCGATATTGCCGACTTCCACTGTGAT-3'), which detected a 323-bp fragment of *B. pseudomallei* capsule gene (Kim et al., 2005). The reaction was carried out in a total volume of 50 µl containing 5 µl of 10 X PCR buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCl), 2.5 µl of deoxynucleoside triphosphates (1 mM each), 2.5 µl of each primers (10 µM each), 5 µl of sample and 5 unit of *Taq* DNA polymerase. The template DNA was initially denatured at 94°C for 5 min. The amplification procedure comprised 40 cycles at 94°C for 30 s (denaturation), 60 ° C for 30 s (annealing) and 72° C for 45 second (extension). PCR using *B. pseudomallei* DNA that prepared by using proteinase K digestion technique

(2 pg/μl) was performed as an internal positive-control. In a negative control PCR was run without *B. pseudomallei* DNAs (non-infected mice and distilled water).

Amplified products were analyzed by electrophoresis in 1.5% agarose gel (Pharmacia, Sweden) using 1 x TBE buffer, pH 8.0 (0.089 μM Tris-HCL, 0.089 μM boric acid, 0.002 μM EDTA) at 100 V for 30 min and was visualized by ethidium bromide staining.

3.4 Determination the kinetic of specific antibody in plasma by ELISA

3.4.1 Antigen preparation

The culture filtrate antigen (CF) of *B. pseudomallei* A_2, which contains multiple proteins and polysaccharides was prepared in Modified Proskauer and Beck medium (MPB medium) as described previously (Wongratanacheewin et al., 1995). Briefly, the bacteria were cultured in MPB medium for 3 weeks at 37° C. The culture-filtrated antigens were collected by centrifugation at 12,800g, 4° C for 20 minutes (Beckman, USA) and filtered through a 0.45 μm membrane. The antigens obtained were then dialyzed thoroughly against PBS three times of and kept frozen at -20° C until used.

3.4.2 ELISA for specific antibody

The ELISA method was performed as described previously (Wongratanacheewin et al., 1995). The 96 wells plates were coated with 100 μl of bacterial culture filtrated antigens at 10 μg/ml in carbonate buffer pH 9.6 and incubated overnight at 4°C. The plates were then washed three times with 0.05% (vol/vol) Tween-20 (Sigma, MO, USA) in 0.15 M phosphate buffer saline, pH 7.4 (PBS-Tween). Each time the washing fluid was left in the wells for 3 minutes. The plates were dried and blocked with 5% skim milk in PBS for 60 minutes at 37°C and then similarly washed prior to adding the samples. Plasma samples were diluted 1:100 in PBS-Tween containing 1% skim milk and 100 μl of each dilution were added. The plates were incubated at 37°C for 2 h. After the plates were washed 3 times with PBS-Tween, 50 μl of horseradish peroxidase-conjugated rabbit antibody to mouse total Ig diluted 1:1000 in 1% Bovine serum albumin in PBS-Tween were added to each well. The plates were then incubated at 37°C for 60 minutes. After washing for 3 times, 100 μl of substrate (R&D system ,USA) were added to each well. The

plates were incubated in the dark at room temperature, and the reaction were stopped after 25 minutes by the addition 50 µl of $2NH_2SO_4$ to each well. The optical density was read at 450 nm in an ELISA reader (TECAN; Synrise Instruments Inc, CA, USA). A positive reference plasma was also included in every plate to correct for day-to-day and plate-to-plate variations that might be present.

Anti- *B. pseudomallei* were assayed, when the average of the OD reading of the test sample was greater than that of the mean of negative controls plus two standard deviations (0.378+0.096), the tested sample was considered to be positive for the specific antibody.

3.5 Statistical analysis

Data were expressed as means ± standard deviation. The difference in mean of bacterial load and antibody levels at individual time points were analyzed using Independent – Sample T-test within the SPSS version 16.0, with a P value < 0.05 considered significant.