CHAPTER III

An approach to isolating *in vivo* expressed genes of *Burkholderia pseudomallei* using IVIAT

Materials and methods

3.1 Bacterial strains

Twenty *B. pseudomallei* strains were collected from blood culture specimens by standard methods, stabbed on semisolid nutrient agar and overlayered with sterile paraffin oil, and placed as stock tubes at 4°C refrigerator until use [23].

3.2 Melioidosis sera

Sixteen serum samples were obtained from hospital in-patients with positive blood cultures for *B. pseudomallei*. The sera were kept in a –80°C freezer until use.

3.3 Normal control sera

Twenty normal serum samples were obtained from healthy donors blood of the blood bank of Thai Red Cross Society.All were nega for SIDa, HBstive All were negative for AIDs, HBs Ag and syphilis. The sera were kept at –80°c freezer until use.

3.4 Irradiated *B. pseudomallei* cell.

A single fresh isolated colony of *B. pseudomallei* was inoculated to 50 ml of LB broth. The broth was incubated at 37°C in a shaker incubator overnight and 5 ml of the culture was inoculated into 5 liters of LB broth. After incubation

at 37°C overnight, total plate count was performed to estimate the total number of bacteria.The cells were then centrifuged at 3,000g for 30 minutes at 4°C. The pellet was resuspended in 5 ml of PBS pH 7. Gamma irradiation was kindly operated by office of Atomic Energy for Peace(OAEP), Thailand.The sample was diluted with PBS to a final volume 50 ml. Sodium azide was added to the sample at .02% final concentration.

3.5 Serum absorption

3.5.1 Membrane preparation

Whole cell lysate of *B. pseudomallei* was prepared by using French pressure. Irradiated *B. pseudomallei* cells at a concentration of 10^{11} cfu/ml were put in the chamber of a French press, which was then operated at 2000 lb/in^2, and the resulting homogenous liquid was collected. The process was repeated for 5 times. The lysate was diluted to 1:10 with PBS pH 7. The 90 mm diameter nitrocellulose membrane diameter (Gibco) was incubated with 5 ml of diluted *B. pseudomallei* lysate. The membrane was rotated for 2 hours at 25°C, then put on Whatman filter paper number three to be air dried. When dried, the membrane was washed 3 times with PBS buffer. The membrane was kept in PBS tween (0.025%) at 4°C until use. Another 5 ml of diluted *B. pseudomallei* lysate was put on a heat block at 100°c for 10 minutes. Heat denature cell lysate was then coated onto membrane by the same process as above.

Table 1 Melioidosis patient data

No	Sex	Age	Primary disease	Underlying disease
1	M	72	Pneumonia	Septic shock, ARF
2	M	52	Pneumonia	DM, dead
3	F	55	Septic shock	Lt. Shoulder joint
4	M	30	Open fracture Lt. Leg	-
5	M	45	Renal failure	DM, dead
6	M	33	Pneumonia	ARF
7	F	45	Melioidosis	DM, Renal
8	F	58	Recovery case, admit hospital for DM	
9	M	58	Melioidosis	DM, hyperthyroid
10	M	28	-	Pus Lt. Leg +ve
11	M	58	CRF ; transient Melioidosis	
12	M	67	Coronary artery disease	DM, Sepsis
13	M	53	Melioidosis	DM, Anemia
14	F	64	Splunic abscess	-
15	M	41	Iver abscess, Synovial fl	DM
16	M	-	Septicemic melioidosis	ARF

3.5.2 Serum absorption process

One hundred microlitres of each melioidosis serum (total 16) was pooledtogether in one tube. The serum was absorbed with three absorbents : whole *B. pseudomallei* cell , French press whole cell lysate coated nitrocellulose membrane, heat –denatured french press whole cell lysate coated nitro-cellulose membrane. The pooled serum, altogether 500 microlitres, was mixed with 10^{11} cfu/ml of *B. pseudomallei* whole cells in a microcentrifuge tube. The mixture was gently rotated at 4°C , then centrifuged at 4,000 g for 10 minutes. The serum was collected and the absorption process was repeated with new *B. pseudomallei* whole cells for another 4 rounds. The absorbed serum was transferred to the centre of a nitrocellulose membrane that was coated with French press whole cell lysate. The plate was gently rotated at 4°C overnight. The absorbed serum was separated from the membrane and this step repeated for another round with a new *B. pseudomallei* lysate coated nitrocellulose membrane. Finally, the absorbed serum was transferred to the nitrocellulose membrane that was coated with denatured *B. pseudomallei* lysate. The process was forwarded as previous nitrocellulose membrane. The absorbed serum was repeated for another round with new coated heat French press whole cell lysate. The absorbed serum, 20 microliters altogether, was collected from each absorption step (9 samples). These samples were analyzed for ELISA titre against the French press whole cell lysate. The absorbed serum was kept at 4°C with 0.05%sodium azide .

3.6 Enzyme Linked Immunosorbent Assay (ELISA)

An ELISA was performed following the method of Ebersole[73]. Whole cells of *B. pseudomallei,* about 10^{11} cfu, were French pressed at 2,000 lbs/in^2 5 times. The homogenous suspension was mixed with 25 ml of coating buffer . One hundred of microliters of antigen suspension was added to each well of

microtiter plate and incubated at 37°C for one hour and then at 4°C overnight. After washing with PBS- Tween, the serum was diluted with buffer dilutent in the well and incubated at 25°C for one hour. After washing, the secondary antibody, peroxidase-conjugated goat anti-human affinity purified immunoglobulin (Cappel, ICN), reactive with all classes of human immunoglobulin at dilution 1:5000, was added to each well except for one row and incubated at 37°C for one hour. The plate was washed 5 times with PBS Tween (Appendix A) and 5 times with PBS buffer. After washing, the reaction was stopped by adding 100 microliters of the developer to each well, then incubating at 25°C for 20 minutes. The plate was read by using an ELISA reader machine.

3.7 Genomic library construction in pET 30a

3.7.1 *B.pseudomallei* DNA isolation

Isolation of *B. pseudomallei* chromosomal DNA by using the "Wizard genomic DNA purification kit" (promega) as follow. An isolate of fresh colony of *B. pseudomallei* was inoculated to 5 ml of LB broth, then incubated at 37°C overnight in a shaking incubator. One ml of the culture was centrifuged at 13,000 g for 3 minutes. The pellet was resus-pended with 600 microliters of nucleic lysis solution. The microcentrifuge tube was incubated at 80°C for 5 minutes and then cooled to room temperature. Three microlitres of Rnase solution was added to the cell lysate. The tube was inverted 25 times and incubated at 37°C for 30 minutes. Two hundred microliters of protein precipitated solution was added to the tube, and the tube was vigorously mixed for 20 seconds, then placed on ice for 5 minutes. The mixture was centrifuged at 13,000 g for 3 minutes. The supernatant was transferred to a new microcentrifuge tube containing 600 microliters of room temperature isopro-

panol. The tube was gently mixed and centrifuged at 13,000 g for 3 minutes. The pellet was air dried. Six hundred microliters of 70% ethanol was added to a pellet and gently inverted several times. The tube was centrifuged at 13,000 g for 3 minutes and air dried. Finally, the pellet was resuspended in 100 microliters of double distilled water and kept at 4 ^{o}C refrigerator.

3.7.2 DNA fragmentation

Fifty microliters of DNA from each of twenty *B. pseudomallei* strain were pooled together. The mixture was concentrated to 200 microliters by using Microcon YM30 (Amicon). The pooled DNA was siphoned to a column of a Gene Machine (Hydroshear), which had a calibrated shearing speed to get the DNA fragment in range 1-2 Kb. The sheared DNA was loaded into 1% low melting point gel, Nusieve GTG (FMC, bioproducts) in tris-acetate EDTA (TAE) (Appen-dix A) with 1 Kb DNA marker . Ethidium bromide was added to the gel at a final concentration 0.5 micrograms/ml, and subjected to 70 volts for 30 minutes. The DNA band was visualized by using long wave UV in the dark room. The 1-2 Kb band range was removed and placed in a 1.5 ml microcentrifuge tube, which was incubated at 70^{o}C for 20 minutes in a heating block. Then 10X of beta-galactase buffer was added to the final 1X and mixed in a vortex. Then one unit of beta-galactase (New England Biolabs) per 250 mg of the melted agarose was added and incubated at 41^{o}C overnight. The melted solution was loaded on a centrifugal filter device Microcon YM10 (Amicon). The solution was adjusted to 2 ml with 20% isopropanol. The tube was centrifuged at 3,000 g for 30 minutes with a fixed angle centrifuge, then the filter was moved to a new filter cup. 1.8 ml of double distilled water was added to the filter and centrifuged at 3000 g for 30 minutes. The filter was inverted, moved to a new filter cup, and centrifuged at 2,000 g for another 2 minutes. The resulting DNA solution was kept in the refrigerator .

3.7.3 BL21DE3 competent cells preparation

An isolate of fresh single colony of *E. coli* strain BL21DE3 was inoculated in 10 ml of LB broth, which was then further incubated at 37°C in a shaking incubator overnight. The culture was then inoculated in 1 litre of LB broth, which was incubated at 37°C in a shaking incubator until the OD_{600} of the culture was 0.5. The broth was placed on ice for 30 minutes, then centrifuged at 4,000 g for 6 minutes at 4°C. The pellet was resuspended in one litre of cold distilled water. The suspension was centrifuged at 4,000 g for 6 minutes at 4°C. The pellet was resuspended in 20 ml of ice cold 10% glycerol. The suspension was centrifuged at 4,000 g for 6 minutes at 4°C. The pellet was resuspended in 2 ml of ice cold 10% glycerol. The competent cell suspension was divided into aliquots of one ml and stored in the freezer.

3.7.4 Plasmid pET30a isolation

The plasmid was isolated by using Wizard plus Minipreps DNA purification systems(Promega). An isolated colony of *E. coli* BL21DE3 that contained pET30a was inoculated to 5 ml of LB broth. The culture was incubated at 37°C overnight in a shaking incubator. The tube was centrifuged at 10,000 g for 10 minutes. The pellet was resuspended with 300 microlitres of cell resuspension solution. Three hundred microliters of cell lysis solution was added to the mixture and mixed by inverting the tube 4 times. Then 600 microliters of the neutralization solution was added to the tube and mixed by inverting the tube for 4 times. The mixture was incubated at room temperature for 10 minutes. The tube was centrifuged at 10,000 g for 5 minutes. The supernatant was transferred to the barrel of the minicolumn/syringe assembly containing 1 ml of resin. The supernatant fluid was pushed through the minicolumn by the syringe plunger. A 2 ml solution of 40% isopropanol/4.2 M guanine hydrochloride (Appendix A) was put in the column and pushed through

the minicolumn resin. The minicolumn was moved to a new syringe. Two milliliters of a wash solution was put in the column and pushed through as previously. The minicolumn was moved to a 1.5 micro-centrifuge tube, centrifuged at 10,000 g for 2 minutes, then transferred to a new microcentrifuge. Fifty microliters of nuclease-free water was added to the column, which was finally centrifuged at 10,000 g for 20 seconds. The filtrate was kept at $4^{o}C$ refrigerater

3.7.5 Agarose gel electrophoresis

Horizontal agarose gel electrophoresis of DNA was carried out via standard methods[74]. The gels consisted of 1% (w/v) agarose (Sigma) in TAE buffer (Appendix A). DNA samples were mixed with 0.1 volume of electrophoresis loading buffer (Appendix A) prior to loading on the gel. Standard DNA marker was co-electrophoresed as a size standard. Gels were run submerged in TAE buffer. Gel was electrophoresed at 70 volts for 1 hour. Following electrophoresis, gels were stained in 5 mg/ml ethidium bromide for 10 minutes. The DNA fragments were visualized on a Biorad multi-analysis (version 1.0.2).

3.7.6 pET30a restriction endonuclease digestion and dephosphorylation.

The plasmid pEt30a was digested using restriction endonuclease enzyme EcoRV (Promega) according to the manufacturer's protocol. Twenty microliters of the reaction volume contained about 1 microgramn of the plasmid DNA, Two microliters of EcoRV buffer(10X), 2 microliters of BSA (10x), 1 microlitre of EcoRV (10 unit/ microlitre), and ddH_2O. After incubation at $37^{o}C$ for 1 hour, the tube was put on heat block at 80 C for 20 minutes to inactivate the activity of the enzyme. Then the 250 microliters of ddH_2O was added to the mixture, and the volume of the reaction concentrated to 20 microliters by using microcon YM30 (Amicon). The digested blunt end of the plasmid was forwarded to the dephosphorylation reaction. The 50-microliter reaction volume contained 20 microlitres of digested plasmid, 1 microliter of calf intestinal alkaline phos-

phatase(CIP)(5 unit/microliter) (Promega), 5 microliters of CIP buffer(10x) and 24 microliters of dd H_2O. The reaction tube was incubated at room temperature for 30 minutes, then 445 microliters of ddH_2O and 5 microliters of 0.5 M EDTA were added to the reaction tube. The tube was incubated at 56^{O} C for 30 minutes, then an equal volume of phenol-chloroform-isoamylalcohol (500 microliters) was added with vigorous mixing. After centrifugation at 10,000g for 3 minutes, the aqueous layer was collected. An equal volume of chloroform was added to the aqueous layer with vigorous mixing. The volume of the extracted layer was concentrated to 10 microliters by using a Microcon YM30 (Amicon). The collected dephosphorylated digested plasmid was kept at 4^{O}C refrigerater for ligation step.

3.7.8 Ligation

The ligation reaction volume of 20 microliters contained 1 microliter of dephosphorylated plasmid, 0.5 microliters of sheared DNA fragment (~40 ng), 2 microliters of ligation buffer (10x), 3 microliters of T4 DNA ligase (3 units) (Promega) and 13.5 microliters of dd H_2O. The ligation reaction was incubated at 16^{O}C for 16 hours, at which time 200 microliters of distilled water was added to the mixture. The reaction volume was concentrated to 10 microliters by using a Microcon YM30(Amicon). The filtrate was collected for the transformation step.

3.7.9 Electroporation

The standard protocol was followed[75]. The competent cell BL21DE3 was thawed and put into a cold 1.5 ml polypropylene tube, 40 microliters gently mixed with 5 microliters of ligation mixture, and the mixture left on ice for about 1 minute. The mixture was then transferred to a cold electroporation cuvette (0.2 cm, Biorad). The Gene Pulse 2 (Biorad) was set to 25 microfarad capacitance, 25 KV/cm and the pulse controller unit to 200 ohms. Then one pulse was applied to the cuvette tube. The time constant was 4.0-4.8 ms after the reaction. Then one ml of SOC medium (room temperature)(Appendix A) was immediately

added to the cuvette, and the culture suspension was gently pipetted to a new 13x100 mm polypropylene tube. After incubation at 37°C for one hour with shaking, the culture suspension was plated out at 1:10-1:100 dilution on LB agar containing kanamycin (50 mg/ml). The plate was incubated at 37° C overnight. The efficiency of transformation was calculated. The genomic library was divided into aliquots with 10% glycerol at their final concentration and kept in the –80 °C freezer.

3.8. Genomic library immunoscreening

The genomic library was thawed and spread on BHI agar containing Kn (50 mg/litre) to obtain a plate with approximately 200 colonies after incubation at 37° C overnight. The clones on the plate were transferred to another two plates of BHI agar with Kn (50 mg/litre) and IPTG(250mg/ml) with a replica machine. After incubation at 37° C for 5 hours, the plates were put in a jar with chloroform for 15 minutes. The nitrocellulose was gently put on the surface of the plate and incubated at room temperature for 15 minutes. The nitrocellulose membrane was gently washed in phosphate buffer saline (PBS) containing 0.025% tween-20 at room temperature, 5 minutes, three times. Then the membrane was blocked in PBS-tween-5% fat-free powdered milk at 4°C overnight for incubation. After washing 3 times with PBS-tween, the membrane was incubated with 5 milliliters of absorbed serum and an unabsorbed serum at a dilution of 1:5000 and incubated at room temperature for 1 hour. After washing 3 times with PBS-Tween, the membrane was incubated with peroxidase-conjugated goat anti-human affinity purified immunoglobulin (Cappel, ICN) at 1;5000 dilution for one hour. After washing 3 times with PBS-Tween. The ECL reagent (Amersham) was added to the membrane and the chemiluminescent reaction was detected by using hyperfilm (Amersham) in a darkroom. The reactive clone position in the

master plate was marked , picked up with a sterile needle and added to the BHI broth contaning Kn (50 mg/litre). The culture was incubated at 37^0 C overnight and forwarded to further investigation.

3.9 Colony dot blot

An overnight incubated BHI broth of positive clones was centrifuged at 3,000 g for 5 minutes. One microliter of the pellet was spotted on BHI agar containing 250 mg/litre of IPTG and Kn (50 mg/litre). The plate was incubated at 37° C for 5 hours. The method of immunoscreening was repeated as in protocol 3.8. A colony that would give a negative reaction from the immunoscreening was included in the colony dot blot for a negative control. The confirmed positive clones were streaked on nutrient agar for further analysis.

3.10 Clone characterization

The confirmed positive clones were analyzed for their insert size, nucleotide sequence data and bioinformatic. The plasmid was isolated and purified from the positive clone by using Wizard^R plus miniprep (Promega) as described in protocol 3.7.4. The inserted DNA size was estimated by performing gel electrophoresis as described in protocol 3.7.5. The migration distance of the isolated plasmids was compared with the DNA marker and the vector without an insert. The nucleotide sequence data were obtained by using T7 and S tag primer (Novagen) with Big dye chemistry on an Applied Biosystem model 377 automated DNA sequencer. The sequence data were analyzed by Macvector V 7.0.1 for a unique insert, open reading frame, start/stop codon, signal sequence such as ribosome binding sites, transcription termination sequences. The sequences were also surveyed for similarity in the GenBank data by using the BLAST program.

3.11 Protein antigen over expression study

The standard protocol for protein expression was followed [76] with some modifications. The positive clones were inoculated into 2 ml of BHI broth containing 50 mg/litre of Kn. After incubation overnight at 37° C, the culture was centrifuged at 3,000 g for 5 minutes. The pellet was resuspended in 2 ml of BHI broth containing 50 mg/litre of Kn and 250 mg/litre of IPTG. The culture tube was incubated at 37° C in a shaker incubater for 5 hours. After centrifugation at 3000 g for 5 minutes, the pellet was resuspended in 100 microlitres of 5x sample buffer and incubated on a heat block at 100° C for 10 minutes. The sample was centrifuged at 10000 g for 5 minutes at 4° C. The protein suspension was further analyzed with SDS-PAGE and western blot.

3.12 SDS-PAGE analysis

The protein suspension was fractionated by SDS-PAGE as described in a standard protocol [76]. A 83x73x0.75 mm (wxhxt) sandwich was used in a miniprotein 3 (Bio-rad) slab gel apparatus. The resolving gel consisted of 12.5% (w/v) acrylamide containing 375 mM Tris-HCl pH8.8, 0.1% SDS and 2% glycerol. The polymerisation was initiated by the addition of ammonium persulphate (APS, Sigma) and N,N,N,N-tetramethylene-ethylenediamine (TEMED, Sigma) both to a final concentration of 0.05%(w/v) and 0.005% (v/v) respectively. The 5% acrylamide stacking gel containing 125 mM Tris-HCl pH 6.8 and 0.1% SDS was polymerised by the addition of APS and TEMED to a final concentration of 0.075 % (w/v) and 0.001% (v/v) ,respectively. The 5-10 microliters of treated protein sample was loaded in the well of the stacking gel. Electrophoresis was performed in SDS-PAGE running buffer at 100 mA for 30 minutes or until the dye front reached the bottom of the gel. Following SDS-PAGE, the gels were processed through western blot analysis or incubated in coomassie gel stain (Appendix A) at room temperature for one hour with agitation. Gels were then

destained in destain solution (Appendix A) overnight. The gels were dried on 3MM paper (Whatman) using a vaccum slab gel dryer for 2-3 hours at 60 ^{0}C.

3.13 Western blot analysis

The standard protocol for western blot was followed[74]. The polyacrylamide gel of the expressed protein and blotting paper (Biorad) were soaked in the transfer buffer (Appendic A) for at least 10 minutes. The polyvinylidene fluoride (PVDF, polyscreen ^{R}) transfer membrane was equilibrated in 100% methanol for 15 seconds, distilled water for 2 minutes and transfer buffer for 5 minutes. The proteins in the acrylamide gel were transferred to the PVDF membrane by using a semi-dry electrophoretic transfer cell (Trans-blot^{R} SD, Biorad). Three pre-soaked, thick blotting papers (Biorad) were placed on the platinum anode of the machine. Then the pre-wetted PVDF was put on the top of the filter paper. The equilibrated acrylamide gel was placed on the center of the PVDF. Another pre-soaked blotting paper was put on the top of the gel, after carefully removing the air bubbles from between the gel and filter paper. The cathode was carefully placed on the stack, then the power supply was turned on at 20 volts for 30 minutes. Following this process, the acrylamide gel was stained with coomasie blue to determine the protein retention on the gel. The PVDF membrane was gently washed in PBS Tween(0.025%) at room temperature for 5 minutes, then the membrane was blocked in PBS tween-5% fat-free powdered milk at 25^{o} C for one hour. After washing 3 times with PBS-Tween, the membrane was incubated with 5 millilitre of absorbed melioidosis serum at dilution 1:2,000 and incubated at room temperature for 1 hour. After washing 3 times with PBS-tween, the membrane was incubated with peroxidase-conjugated goat anti-human affinity purified immunoglobulin (Cappel, ICN) at 1:5000 dilution for one hour. After washing 3 times with PBS-tween, the ECL reagent (Amershamp) was

added to the membrane and the chemiluminescent reaction was detected by using hyperfilm in a darkroom.

3.14 Inclusion body preparation

The standard protocol[77] was followed with some modifications. A fresh isolated colony of Bp 23 positive clone was inoculated in 5 ml of LB broth containing Kn (50 mg/litre). After incubation at 37° C overnight with shaking, the culture was centrifuged at 3000g for 4 minutes. The pellet was resuspended in 20 ml of LB broth containing Kn (50mg/litre) and IPTG (2mM) and incubated at 37° C for 5 hours with shaking. After centrifugation at 3000 g for 10 minutes, the pellet was resuspended in 5 ml of IB buffer(Appendix A) containing lysozyme (5 mg/ml) and incubated at 37° C for 10 minutes. The suspension was centrifuged at 5000 g for 10 minutes in fixed angle beckman SS-34. The spheroplast was collected and resuspended in 5 ml of an ice-cold IB buffer containing sodium deoxycholate (Sigma) at a final concentration of 0.1%. The tube was incubated on ice with occasional mixing for one hour. Then $MgCl_2$ and DNaseI were added to the mixture at a final concentration of 8 mM and 10 micrograms/ml respectively. The inclusion body was removed from the suspension by centrifugation at 10,000 g for 10 minutes. The pellet was collected and washed with 5 ml of 1% Triton X by centrifugation at 10,000 for 10 minutes. The washing steps were repeated with 1% Triton X, distilled water (2 times), and IB buffer (one time). The pellet was resuspended in 1 ml of 8M urea and dialysed at 4^0 C overnight in dialysis buffer (Appendix A). Then the protein solution was centrifuged at 10,000g for 10 minutes. The inclusion body in supernatant was processed for SDS-PAGE and western blot analysis.

Results

1. Enzyme linked immunosorbent assay(ELISA)

The ELISA titers against whole cell lysate of *B. pseudomallei* of 16 melioidosis serum and 17 control serum samples were demonstrated in table 1 and Figure 4. All of the melioidosis serum samples had a titer range from 4000 to > 128,000. Four samples had a titer of 4000, equal to those of normal sera that included one American sample. The normal control sera had titer range of 4000-8000.

2. Serum absorption assay

The ELISA titer against whole cell lysate of *B. pseudomallei* of nine absorbed serum samples from the absorption process are demonstrated in Figure 5. The ELISA titer was decreased during the absorption process. After the 6th absorption process, the optical density of the absorbed serum was equal to back ground. The cumulative depletion factor was more than 1000 times, and the titer was reduced from >102,400 to <1:50, as shown in Table 2 .

3. Expressed genomic library construction

3.1 DNA fragment preparation

The pooled *B. pseudomallei* DNA was cut using a hydroshea machine, as demonstrated on 1% agarose gel in Figure 6. The high concentration of DNA with dense banding was in the range 0.5 Kb to 3 Kb. The DNA bands in range 1-2 Kb were cut and reisolated.

3.2 Expression genomic library in pET30 a

The expressed genomic library was constructed in plasmid pET 30 a with a moderate transformation coefficient of about 1.67×10^5 cfu/microgram.

4. Genomic library immunoscreening

About 13,000 recombinant clones were screened using both absorbed and unabsorbed melioidosis sera, as partially shown in Figure 7. Thirty-one positive clones were confirmed by using colony dot blot analysis as demonstrated in Figure 8.

5. Clone Characterization

Eight out of 31 total positive clones were successfully sequenced in both directions with Stag and T7 primer. The insert size ranged from 1-2 Kb. The expressed proteins of 29 positive clones were demonstrated in a 12.5% SDS-PAGE range from 14kDa to 45kDa (some are shown in Figure 9). There were 8 positive clones that had both sequence DNA and SDS-PAGE data (Bp1, Bp22, Bp23, Bp24, , Bp28, Bp29, Bp38, and Bp43).The DNA sequence of the insert at Stag site of each clone was analyzed for an inframed open reading frame by using the Macvector program. The inserted sequences were also searched for similarity against the GenBank sequence data using the BLAST program as demonstrated in table 4.

6. Western blot analysis

There were 19 positive clones, numbered Bp 1, Bp4, Bp 6, Bp 8, Bp 14, Bp 22, Bp 23, Bp 24, Bp 25, Bp 27, Bp 28, Bp 29, Bp 32, Bp 34, Bp 38, Bp 39, Bp 40, Bp 43, and BP 44, which were selected for western blot analysis. Three serum samples, control Thai normal serum (no.16), Stag serum and pooled absorbed melioidosis serum, were used as probes. All of the positive clones reacted with all types of serum used in the experiment as demonstrated in Figure 10.

7. SDS-PAGE and Western blot analysis of Inclusion Body of Bp23

The expressed protein from each step in the inclusion body preparation of Bp23 was demonstrated on 12.5% SDS-PAGE, as shown in Figure 11. The protein content decreased after the washing steps. The western blot was performed using 16 melioidosis serum and 16 normal control sera. One normal control sera was American donor blood which had not been in contact with any melioidosis. Figure 12 demonstrates one inclusion body band that reacted with all of the serum samples.

Table 2 ELISA titers of sera against French-pressed whole cells of *Burkholderia pseudomallei*

patient no	titer	control no	titer
1	> 128,000	1	4000
2	4000	2	4000
3	4000	3	4000
4	4000	4	4000
5	> 128,000	5	4000
6	4000	6	4000
7	> 128,000	7	4000
8	> 128,000	8	4000
9	4000	9	4000
10	> 128,000	10	4000
11	> 128,000	11	4000
12	4000	12	4000
13	32,000	13	4000
14	64,000	14	4000
15	> 128,000	15	4000
16	> 128,000	16	4000
		17	4000
		18	4000
		19	4000
		20	4000
		21*	4000

* American normal serum

Table 3 ELISA titer of pooled melioidosis serum after absorption process

Absorption	ELISA titer*	Depletion factor	Comulative depletion factor
None	> 1/128,000	0	0
whole cells	1/800	> 128x	$> 10^2$
Immobilized French-pressed	< 1:50	10x	$> 10^3$
Immobilized heat-denature French-pressed	< 1:50	< 1x	$> 10^3$

* highest dilution at which a signal was detected

Table 4 List of isolated IVIAT clones

Clones	Expressed protein	Inframed ORF (bp)	BLAST X		
1 A	18KDA	225	hypothetical protein *B.fungorum*	S = 118,	E = 3e-26
22 A	25KDA	390	hypothetical protein *Ralstonia metallidurans*	S = 64,	E = 1e-09
23 A	45KDA	>570	hypothetical protein *B.fungorum*	S = 239,	E = 2e-62
24 A	14KDA	-	hypothetical protein *B.fungorum*	S = 183,	E = 1e-45
28 A	<14KDA	96	hypothetical protein *P.fluorescens*	S = 111,	E = 6e-24
29 A	20KDA	-	hypothetical protein *B.fungorum*	S = 171,	E = 5e-42
38 A	20KDA	-	hypothetical protein *B.fungorum*	S = 175,	E = 2e-43
43 A	21KDA	180	hypothetical protein *B.fungorum*	S = 114,	E = 4e-25

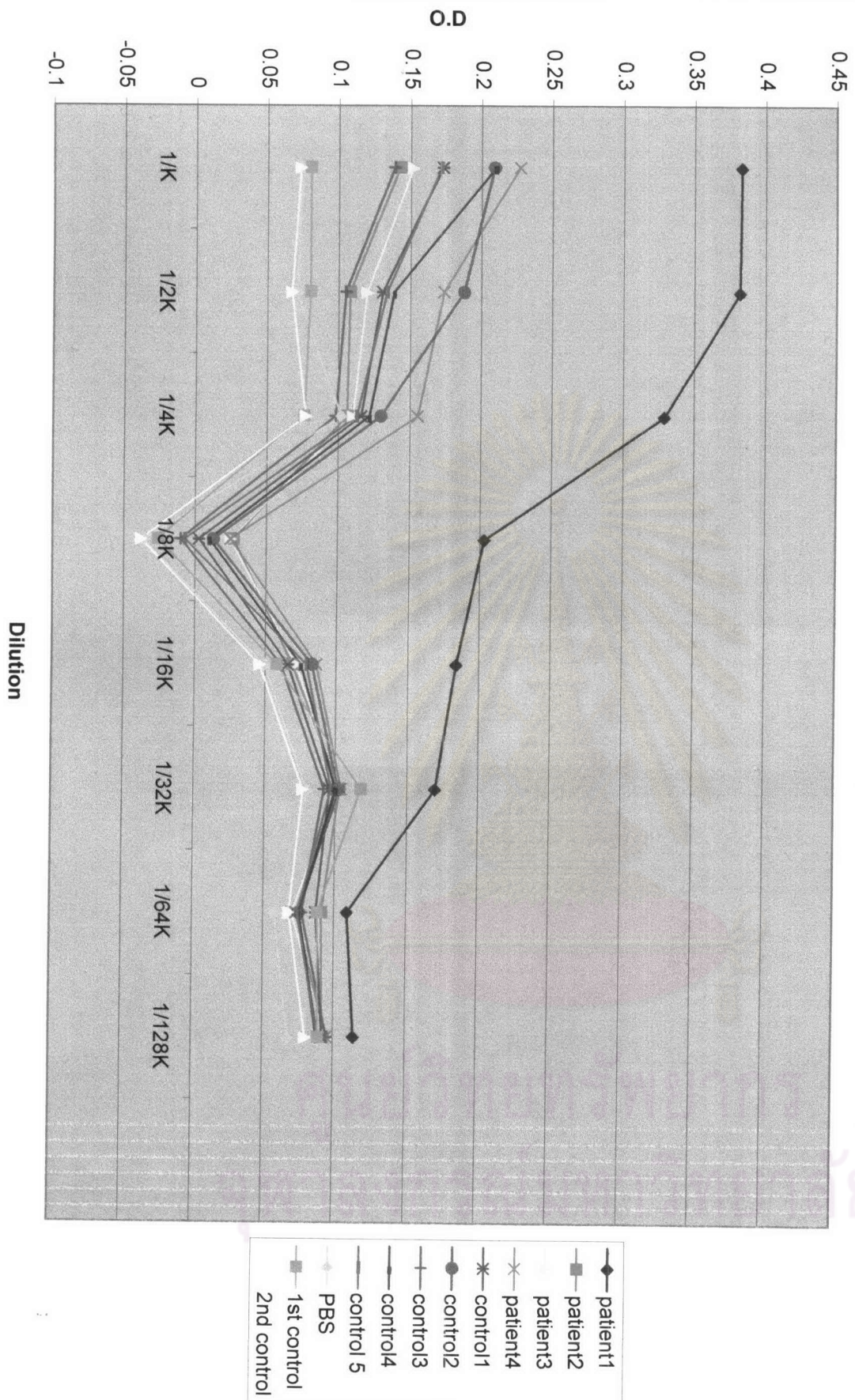

Figure 4 ELISA assay of representative melioidosis patients ' and control sera.

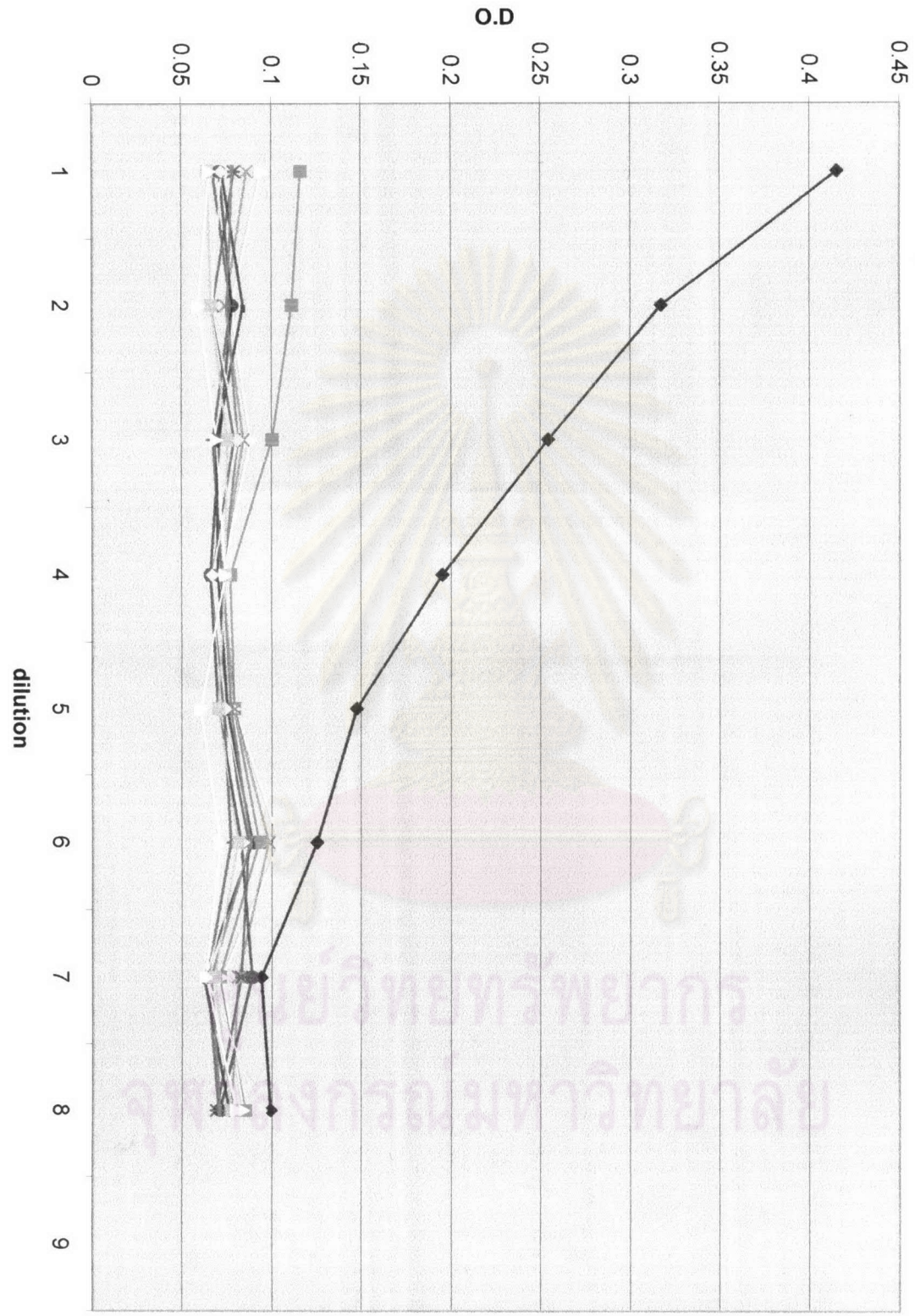

Figure 5 Serum absorption assay of pooled melioidosis serum.

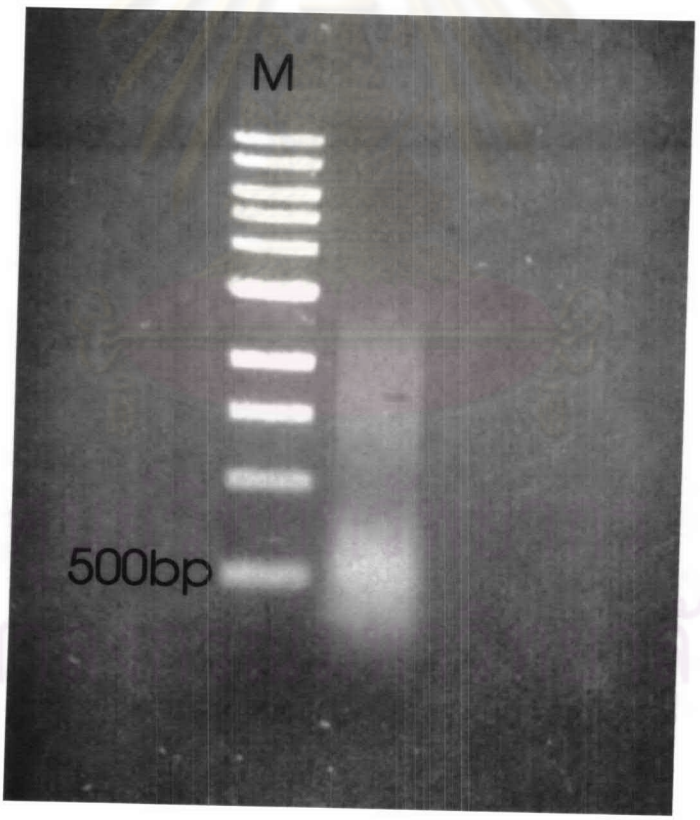

Figure 6 Generation of DNA fragments by using hydroshea

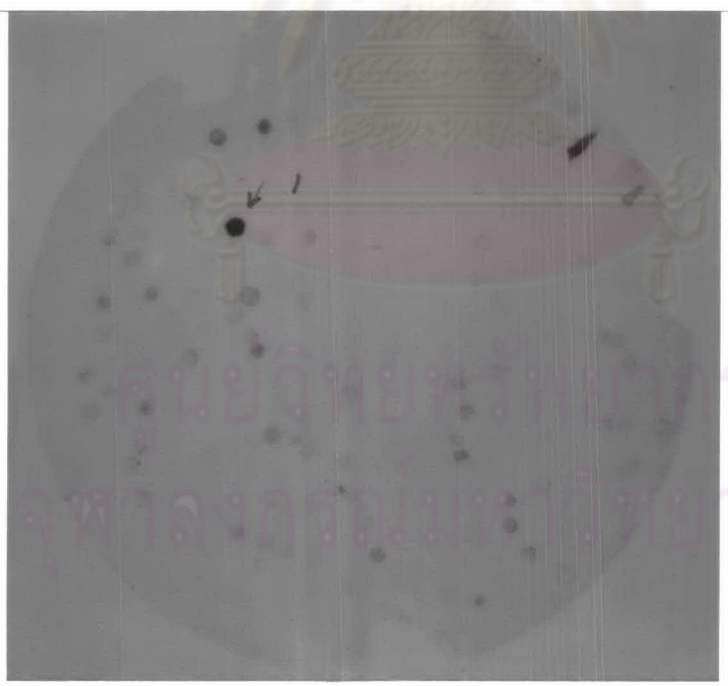

Figure 7 Immunoscreening plate

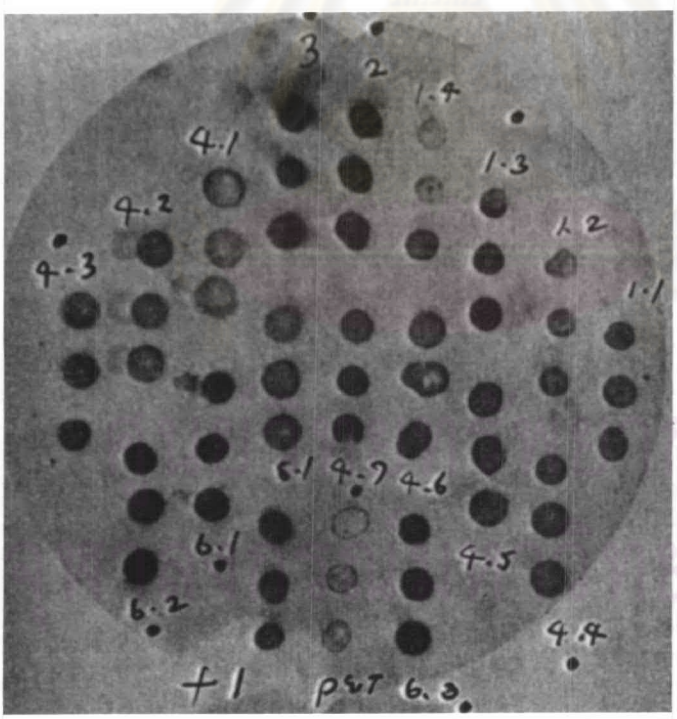

Figure 8 Colony dot blot analysis

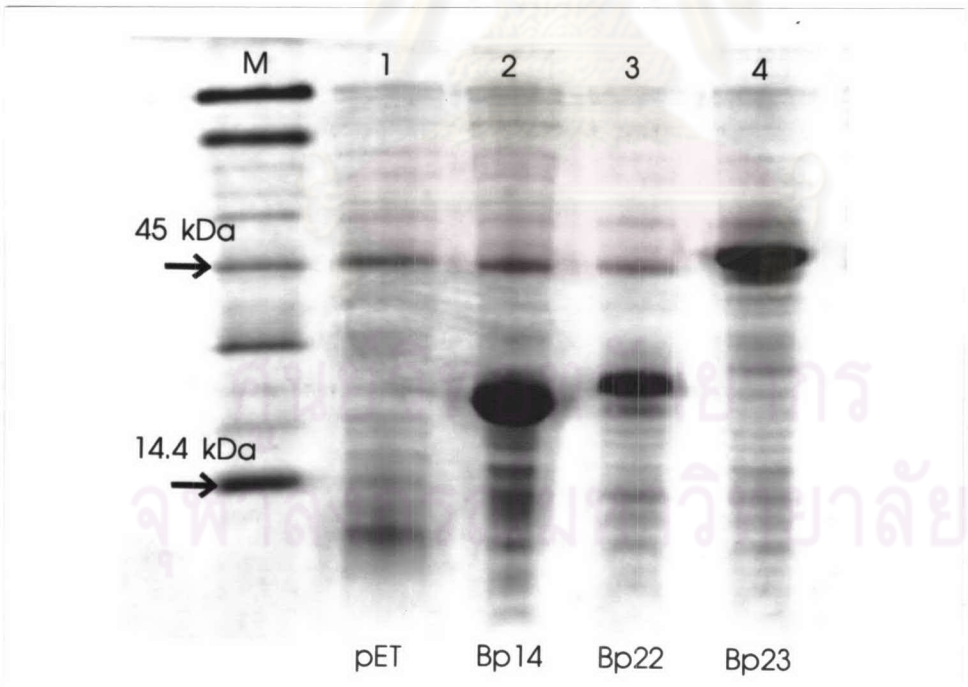

Figure 9 SDS-PAGE analysis of representative positive clones.

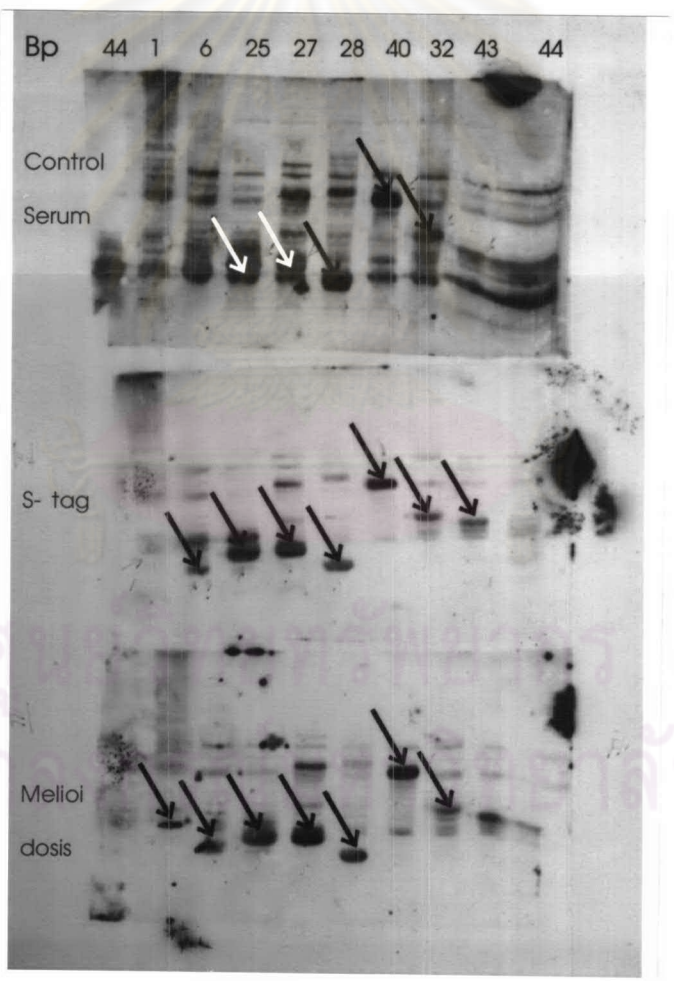

Figure 10 Western blot analysis of representative positive clones

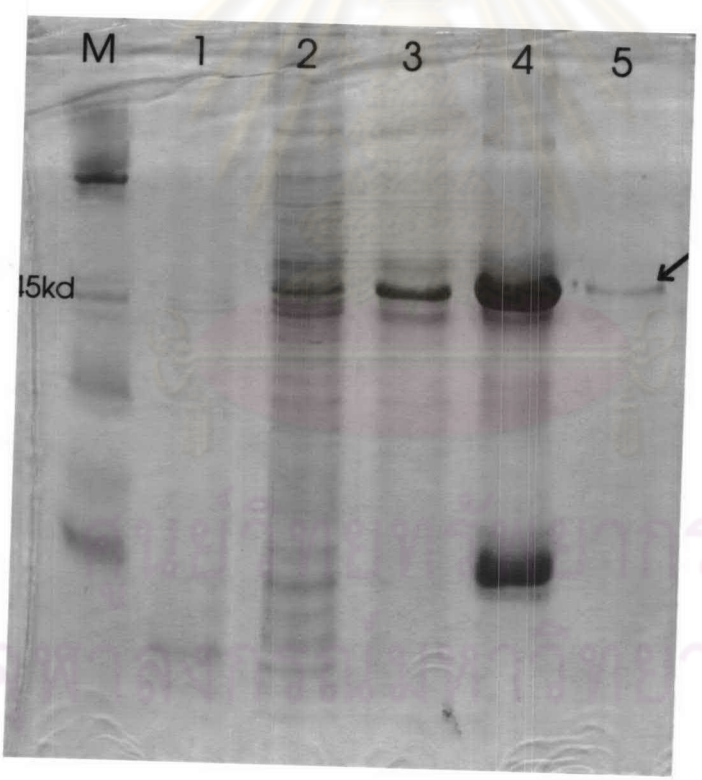

Figure 11 SDS-PAGE analysis of inclusion body of clone Bp23

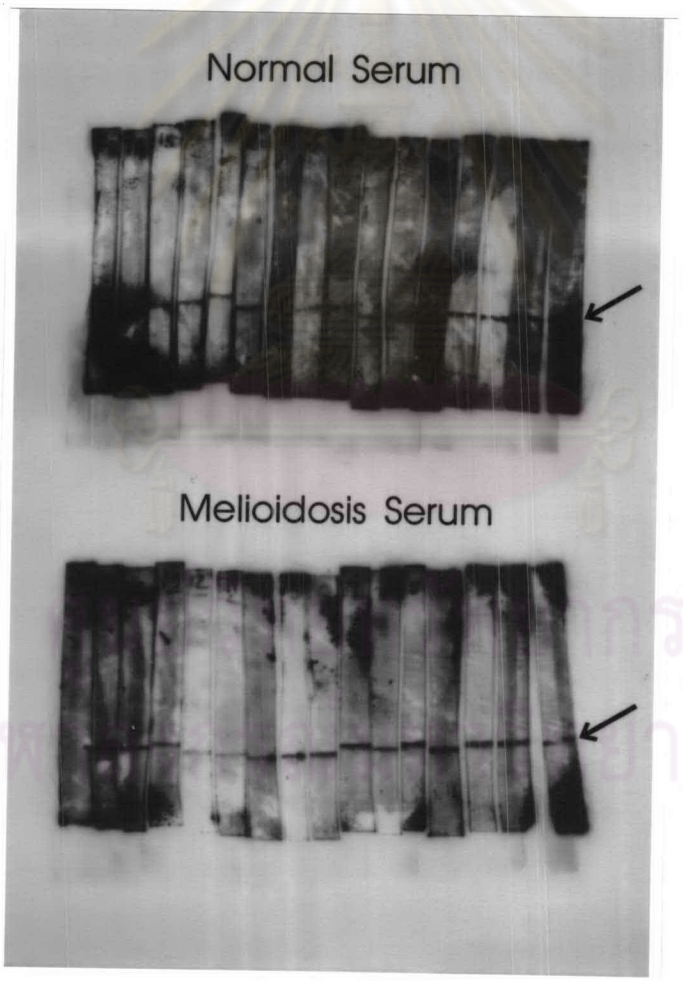

Figure 12 Western blot analysis of inclusion body of clone Bp23.

Discussion

The genomic library screening data from this experiment demonstrate that the IVIAT approach may be an alternative method for isolating *in vivo* expressed genes of melioidosis even though the well known virulent genes were not found in this particular search. This view is supported because only 8 out of 31positive clones were successfully sequenced, and also because of the high sensitivity of the chemiluminescent detection system in combination with a good expressed genomic library construction. The *B. pseudomallei* DNA fragments of 1-2 kb that were prepared using the hydroshear machine could be directly ligated with dephosphorylated plasmid vector, as in previous reports [78,79]. However, the ligation reaction required a 10-fold higher concentration of ligase enzyme than under normal conditions. The application of microcon YM10 during the library construction also increased the transformation yield, perhaps because the contaminated ions could be eliminated from the reaction mixture, preventing a spark reaction during electroporation.

The original IVIAT approach recommended subcloning the inframed open reading frame of the positive clone through PCR, then the protein from the subclone can be used to produce polyclonal antibodies. The clinical specimen from the patient was probed with polyclonal antibodies to verify the *in vivo* induced antigen[16]. After a positive clone was obtained, our approach differed from the original IVIAT in that the combination of SDS-PAGE, open reading frame analysis with the Macvector 7.0.1, and a gene finding program by GeneMark, an heuristic approach of the DNA sequence with the Stag primer of each clone was used to isolate the full gene of *in vivo* expressed *B. pseudomallei* in the GenBank data. All except the Bp23 clone could produce up to 20kDa of the expressed protein. The DNA sequence with the Stag primer of each clone was enough for analysis of the inframed open reading frame. The

size of inframed open reading frame in combination with the size of the expressed protein on SDS-PAGE were used to estimate the expressed gene size. Then the inserted DNA sequence was analysed for the gene using the GeneMark heuristic approach [80] , similarities searched by using BLAST[81,82]. Normally we could not get the full gene by this technique according to the method of library construction. Then The DNA sequence was blasted against the *B. pseudomallei* genome (from the Sanger web site http://www.sanger.ac.uk). The hit area of the *B. pseudomallei* was extended to both direction (left and right) for approximately 1000 bp, then cut, with the gene searched for using GeneMark program. The suspected expressed full gene was cut and searched for similarity against GenBank data by using BLAST. The full gene size 876 bp of Bp 23 was isolated from the *B.pseudomallei* genome. However, the other clones could not be blasted against this *B.pseudomallei* genome because the percentage of identity was too low to locate their positions on the *B. pseudomallei* chromosome. We did use the same technique to isolate the full gene from the incomplete genome of *B. mallei* (http://www.tigr.org). The reason for these results was that our positive clone, isolated *B. pseudomallei* DNA sequence had a different sequence from the standard strain in genbank. The *B. mallei* organism is very closely related to the bacterium *B. pseudomallei* [83].There have been many reports of bacterial genomes of the same species with different strains in the GenBank, including *E. coli* and *S.enterolitica* [84]. Even though the isolated full genes of *in vivo* expressed clones were not well known virulent genes, this approach has been shown to work very well.

In the western blot experiment, 19 positive clones reacted with normal serum. In comparison with the colony dot blot experiment, the plasmid pET30 a without an inserted clone, and some negative clones from immunoscreening, also did not react with the pooled absorbed melioidosis serum. Possible explanation for this contradictory result may cause by a cross reaction between

the *E.coli* host cell (BL21 DE3) and antibodies in normal healthy person. In addition, one American normal serum who had never been in contact with this organism also reacted with the inclusion body of Bp23. There was no *E.coli* BL21DE3 lysate absorption in the absorption process that giving it a high probability to retain some antibodies to the *E.coli* components. Even though some of them may cross-absorbed by using *B. pseudomallei* whole cell and whole cell lysate , they may have reacted with the *E. coli* component.

The IVIAT concept states that after many rounds of absorption with *in vitro* grown bacterial pathogen cells and lysate, all antibodies against *in vitro* produced antigens will be eliminated. On the other hand, only antibodies against *in vivo* produced antigen will be left in the pooled absorbed patient serum[16]. However, even though our modified technique for the full expressed gene worked very well, the IVIAT protocol needs to be reconsidered as follows:

1.The serum absorption process needs to be changed in both the protocol and concept because the quality of the antibodies plays a major role in the positive clone selection.

1.1 The pooled serum needs to be absorbed with the whole cell lysate of the *E. coli* BL21DE3 host cell .Our bodies have been exposed to normal flora in the environment, particularly the GI-tract since we were born, therefore our serum usually contains a certain level of natural antibodies against *E .coli* components and products[85]. If this group of antibodies still exists in the pooled serum at a high level, a false positive clone will be selected .

1.2 The pooled serum may not need to be absorbed with the *in vitro* grown *B. pseudomallei*. Some virulent genes have been reported to be expressed *in vitro* condition [86].Currently, we do not know the number of genes that express both *in vivo* and *in vitro*[65].Following the original IVIAT protocol, this group of genes may be eliminated from the pooled serum

2.The expression vector system needs to be improved. The lambda expression system has been reported to be more useful than expressed plasmid vectors, as having a higher efficiency in library construction, thus a larger number, up to 3,000 recombinant clones could be screened in one plate, making it easier to do a screening.

3.The concepts currently used to approach the *in vivo* expressed genes need to be reviewed. Many housekeeping genes have been reported as *in vivo* expressed genes in various animal models and tissue cultures [65]. Such genes may function by giving support for survival and multiplication in the extremely harsh environment of the host cell. However, only a few of them have been shown to be important for survival using a mutation test [65]. In melioidosis, the incubation period time may range from 2 days to 29 years [87], and people exposed to *B. pseudomallei* may develop different clinical symptoms ranging from asymptomatic to acute fulminant septicaemic melioidosis[1]. This picture supports the complexity of the regulation of virulent genes. On the other hand, the pathogen may express different groups of the genes in each group of the melioidosis patients. If we can design an experiment to identify those groups of genes, the pathogenesis of melioidosis would be more clearly explored. A suitable baseline to see the difference in gene expression may be a comparison between a normal healthy person in an endemic area and melioidosis patients, and the research direction could focus on the group of genes that are expressed only in the melioidosis patient not in the normal healthy person. We can apply this concept to an immunoscreening experiment by looking for positive clones that react only with pooled absorbed melioidosis serum not pooled absorbed normal serum.

The results from this experiment demonstrated some weaknesses with the original IVIAT method. There are some possibilities to isolate *in vivo* expressed genes by using an immunoscreening experiment if the protocol and concept are improved as discussed above.