CHAPTER III

MATERIAL AND METHODS

1. Bacteria

Thirty five strains which were recieved as *P. pseudomallei* were used in this study. Thirty two strains were isolated from human, another two from the environment and the other from one animal. The reference strains used in this study were; *P. pseudomallei* NCTC 4845, *P. aeruginosa* ATCC 27853, *P. cepacia* JCM 5510, *P. stutzeri* JCM 5965, *P. putida* JCM 6160, *P. maltophila* JCM 3801, *V. cholerae* 569 B, *S. typhi* NCTC 781, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923.

All of these strains were shown in Table 4 and were maintained as stock cultures at -70 °C in maintenance medium (Appendix 1) during this study.

2. Identification of *P. pseudomallei*

The forty one biochemical tests (Appendix 2) were performed according to the methods of Cowan & Steel[88] and Weaver[87].

All strains were identified as *P. pseudomallei* by the following characters:

- small gram negative bipolar staining
- polar tuff flagella (multitrichous flagella)

Table 4 Strains of bacteria

Strain number	Species	Laboratory designation	Isolation		
			Year	Source	Place
1	*P.pseudomallei*	CPp 01/85	1985	human blood	Chulalongkorn hospital
2	"	CPp 02/85	1985	"	"
3	"	CPp 02/86	1986	"	"
4	"	CPp 03/86	1986	"	"
5	"	CPp 04/86	1986	"	"
6	"	Cpp 05/86	1986	human sputum	"
7	"	CPp 06/86	1986	human pus	"
8	"	CPp 01/87	1987	human blood	"
9	"	CPp 02/87	1987	human pus	"
10	"	CPp 05/87	1987	human blood	"
11	"	CPp 06/87	1987	"	"
12	"	CPp 07/87	1987	"	"
13	"	CPp 01/88	1988	"	"
14	"	CPp 03/88	1988	"	"
15	"	CPp 04/88	1988	human synovial fluid	"
16	"	CPp 05/88	1988	"	"
17	"	CPp 06/88	1988	human blood	"
18	"	CPp 07/88	1988	human pus	"
19	"	SPp 01/84	1984	"	Songkla Nakarin hospital
20	"	SPp 01/87	1987	"	"
21	"	SPp 02/87	1987	human blood	"
22	"	SPp 03/87	1987	"	"
23	"	SPp 04/87	1987	human pus	Nakornsrithamaraj hospital
24	"	SPp 05/87	1987	human blood	Songkla Nakarin hospital
25	"	SPp 06/87	1987	human sputum	"
26	"	CHPp 01/86	1986	human pus	Bhudhachinaraj hospital (Pissanulok Province)
27	"	CHPp 01/87	1987	human blood	S[illegible]-Kork hospital (Chiangsai province)
28	"	CHPp 02/87	1987	human urine	"
29	"	CHPp 03/87	1987	human blood	"
30	"	KPp 01/87	1987	human pus	Khonkaen hospital
31	"	KPp 02/87	1987	"	"
32	"	KPp 01/88	1988	human blood	Bangphra (Khonkaen)
33	"	DM SO 0732	1987	soil	Khonkaen (province)
34	"	DM SO 0734	1987	"	"
35	"	CowPp 01/88	1988	cow	"
36	"	NCTC 4846	1935	monkey	Singapore
37	*P.aeruginosa*	ATCC 27853	-	-	-
38	*P.cepacia*	JCM 5510	-	-	-
39	*P.stuzeri*	JCM 5965	-	-	-
40	*P.putida*	JCM 5160	-	-	-
41	*P.maltophila*	JCM 3801	-	-	-
42	*V.cholerae*	569 B	-	-	-
43	*S.typhi*	NCTC 781	-	-	-
44	*E.coli*	ATCC 25922	-	-	-
45	*S.aureus*	ATCC 25923	-	-	-

CPp = clinical isolation of *P. pseudomallei* from Chulalongkorn hospital

SPp = clinical isolation of *P. pseudomallei* from hospital in Southern part of Thailand

CHPp = clinical isolation of *P. pseudomallei* from hospital in Northern part of Thailand

KPp = clinical isolation of *P. pseudomallei* from hospital in Northeastern part of Thailand

NCTC = National Collection of Type Cultures (London)

ATCC = American Type Culture Collection (Rockville, Md.)

JCM = Japanese Culture Microbiology

- motile
- positive for
    - oxidase
    - catalase
    - nitrate reduction and gas formation
    - arginine dihydrolysis
    - starch hydrolysis
    - oxidation of glucose, lactose and maltose
    - growth on Mac Conkey
    - growth at 42 °C
- negative for
    - H_2S production
    - indole formation
    - lysine and ornithine decarboxylase
    - growth on 6.5% NaCl

3. Preparation of sonic extract antigens

Sonic extract antigens were prepared from forty five strains of bacteria (as shown in Table 4) by the procedure of Mc Coy[88] with slightly modification:. These antigens were used in SDS-PAGE and immunoblot assay.

Beginning with stock cultures, each organism was subcultured on a 5% sheep blood agar plate and incubated for 18 hrs at 37 °C. One colony of pure cultures was transferred to 50 ml brain heart infusion broth (BHI) and incubated for 18 hrs at 37 °C with constant agitation in an incubator shaker at the speed of 100 rpm/min. Then these broth cultures were subcultured for purity determination. The

bacteria was pelleted by centrifugation at 1250 g for 30 min at 4 °C and washed in sterile normal saline (NSS) three times. After centrifugation, bacterial pellets were suspended in sterile NSS with final concentration approximately 5 mg/ml (wet weight) and kept in -70 °C before use. The bacterial cells were thrawed and disrupted by an ultrasonic disintegrator which was set to operate at 2 min interval. The sonication step was carried out until most intact cells were broken by observing under a light microscope (100x). It took approximately five times. To prevent overheating, this process was performed in 5-min sessions. The probe and attached vial was cooled in ice bath between each sonication session. The sonicated bacterial homogenates were then centrifuged at 12,000g for 20 min in a high speed centrifuge. The pellets were discarded and the supernatants were kept frozen in small aliquots at -70 °C until used. All steps of antigen preparation were carried out at 4 °C. The protein contents were assayed by modified Lowry method[88].

4. Protein determination

The concentration of protien was determined by modified Lowry method[88] A sample was diluted with deionized distilled water (DDW) and then 1 ml of the diluted sample which contained 10 to 100 ug of proteins was added to 3 ml of reagent C (alkaline copper reagent) (Appendix 4.3) and incubated at room temperature for 10 min. The sample was vigorously mixed with 0.3 ml of diluted phenol reagent (Appendix 4.4) and incubated for 45 min at room temperature. Optical density was read at 660 nm on a spectrophotometer. Bovine serum albumin was used as a standard for setting the calibration curve by plotting the protein concentation

against the optical density.

The protein concentration of sonic extract of each bacteria was estimated from the standard curve. (Appendix 4.6)

5. Normal rabbit and polyclonal antisera

1.25 mg protein of the whole cell sonic extract of *P. pseudomallei* NCTC 4845 emulsified with an equal volume of the complete Freund's adjuvant was used to immunize subcutaneously into the legs and backs of one adult rabbit. The preimmunizing sera for antibody to *P. pseudomallei* by IHA was negative (IHA titer below 1:10). 14 and days 21 after initial immunization with 0.625 mg protein were given at the booster doses. The sera collected on day 30 and day 35 and were kept in small aliquot at -20 °C until used.

6. Indirect haemagglutination test (IHA)[90]

6.1 Formalin treatment of sheep red blood cells (SRBC)

The SRBC were washed four times with sterile normal saline (NSS) and then diluted 1:10 in NSS. The erythrocyte suspension was treated with an equal volume of 7.5% formalin in NSS and incubated in a waterbath shaker at 37 °C for 18-20 hrs. The SRBC were washed four times with NSS and finally resuspended in NSS as a 10% suspension (10% formamide treated sheep red blood cell). This suspension was stored at 4 °C.

6.2 Tannic acid Treatment

The 10% formamide treated sheep red blood cells were washed

once in NSS, resuspended as a 2% suspension in PBS pH 7.2 (Appendix 6.2.1) and mixed with an equal volume of tannin solution (Appendix 6.2.3) The mixture was incubated in a waterbath at 37 °C for 30 min. The SRBC were then centrifuged, washed twice in PBS pH 6.4 and resuspended as a 2% suspension in the same buffer. This suspension could be used up to 8 weeks when stored at 4 °C.

6.3 Antigen sensitization

The optimum dilution of sonic extract antigen of *P. pseudomallei* NCTC 4845 for the sensitization with tannic formamide treated SRBC was predetermined by appropriate block titration of two fold dilutions of positive control antiserum from the patient with melioidosis and various dilutions of antigen (sonic extract antigen of *P. pseudomallei* in an equal volume)

The sensitizing dose was the highest dilution of the antigen that effected complete agglutination with the highest serum dilution. The optimum sensitizing dilution of antigen was 1:100. (41.5 ug protein) The maximum IHA titer of the positive control serum was about 1:640.

Sonic extract antigen which was diluted to 1:100 in PBS pH 6.4 was mixed with an equal volumes of 2% suspension of tanned formalin treated SRBC in PBS pH 6.4 and incubated for 1 hr in a 37 °C waterbath. The sensitized SRBC were then washed twice in 1% PBS-R (Appendix 6.3) as 1% suspension.

SRBC treated in the same way with PBS pH 6.4 without antigen,

were used as control cells.

6.4 Performance of the test

Rabbit antiserum and possitive control serum were inactivated at 56 °C for 30 min. and diluted to 1:5 with PBS pH 6.4. The titration of antiserum was perfomed in a microtitration plate Two folds serial dilutions from initial 1:10 were made in 1% PBS-R. The volume in each well was 25 ul. An equal volume (25 ul) of optimally sensitized cell suspension was added to each wells. The serum sample and PBS alone were also tested with 1% control cell and sensitized cell, respectively. The plate was carefully shaken, then covered and left over night at room temperature before read.

The titer was recorded as the reciprocal value of the lowest serum concentration that showed a definite positive pattern when compared with the negative control. Negative controls consisted of unsensitized SRBC plus test serum and sensitized SRBC plus diluent.

7. Preperation of rabbit immunoglobulin

A globulin fraction of rabbit sera was based on the ammonium sulfate procedure[81] as follows.

The pool sera was precipitated for globulin by 33% saturated ammonium sulfate (Appendix 7.1) which was added drop wise to the serum with gentle continuous stiring on a magnetic stirer. The solution was stirred at 4 °C for 3 h. The precipitate was seperated by centrifugation at 10,000 g at 4 °C for 30 min.

After that the precipitation was dissolved in a small volume

of normal saline and repeated precipitation until a colorless supernatant fluid was obtained usually after three precipitations. The final product was dissolved in small volume of 0.01 M phosphate buffer pH 7.2 (PB) (Appendix 7.4) and dialysed aginst several changes of PB at 4 °C until the dialysate was negative for sulfate when tested with barium chloride. Finally the supernatant was seperated by centrifugation at 10,000 g, 4 °C for 30 min. This globulin was collected and aliqouted stored at -20 °C for later use.

8. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE which was used for separating and determinting the molecular weight of the sonic extract components was carried out by the method of Laemmli[92].

8.1 Preparation of sample

The sonic extract components were solubilized in the sample buffer (Appendix 8.8), boiled for 5 min and then centrifuged at 12,000 g for 5 min. The supernatant was then applied to SDS-PAGE or frozened at -20 °C for later use. (The frozen samples must be reheated at 100 °C for 5 min before applying to the gel).

8.2 Preparation of slab gel and electrophoresis

Discontinuous SDS-PAGE was performed on a 16x16x0.1 cm vertical slab gel with 3% stacking gel and 12% resolving gel.

The resolving gel was prepared by mixing the acrylamide stock in 0.375 M Tris-HCl pH 8.8 (Appendix 8.2) containing 0.1% SDS and 0.05 M EDTA as shown in Table 5. The solution was degased

thoroughly; the TEMED and ammonium persulfate were added to polymerize the acrylamide and swirled gently to mix. The solution was pipetted immediately into the prepared gel mould at height of 12 cm., then overlaid carefully with distilled water and let the gels stand for 1 hr at room temperature. The overlaid solution was poured off before preparing the stacking gel. A folded piece of filter paper was used carefully for absorbing any solution which was left without disturbing the gel surface. It was important that the resolving gel surface was dry in order to obtain satisfactory polymerization at the resolving gel interface.

The stacking gel was prepared by mixing the acrylamide stock in 0.125 M Tris-HCl pH 6.8 (Appendix 8.3) which contained 0.1% SDS and 0.05 M EDTA as shown in Table 5. The TEMED and ammonium persulfate solution were added, swirled gently to mix. A 20-well comb was inserted into the top of the gel mould and the gel was left to polymerize completely for about 1 hr at room temperature.

After the gel was polymerized, a small amount of water was added on the top of the gel at the corners. The comb was removed gently by pulling in vertical direction and then rinsed each well to remove any unpolymerized acrylamide. Buffer was pipetted into the mould until all wells were completely tilled and the buffer was then removed by using a syringe with a blunt needle. The gel was mounted in the upper buffer reservoir and inserted into the lower buffer tank. The electrode buffer (Appendix 8.8) was filled in the upper buffer reservoir and lower buffer tank. Electrophoresis was carried out with constant current of 30 mA for 30 min to remove the excession

Table 5 Composition of SDS - Polyacrylamide Gels

solution (ml)	% Acrylamide	
	Stacking gel 3%	Separating gel 12%
Stock acrylamide	1	16
1.5 M Tris - HCL pH 8.8	-	10
0.5 M Tris - HCL pH 6.8	2.5	
10% SDS	0.1	0.4
0.2 M EDTA	0.1	0.4
Distilled water	6.25	12.98
	degas	
TEMED	0.005	0.02
10%$(NH_4)_2S_2O_8$	0.05	0.2
Total Volume (ml)	10.000	40.000

for ion in gel. After the prerun, the upper buffer reservoir and gel removed were from the lower buffer tank, then poured off the electrode buffer from the upper buffer reservoir which was removed from gel accordingly. All wells were filled with electrode buffer and each sample was applied with a required volume into gel by using a microtiter syringe. The gel was again mounted to the upper buffer reservoir, inserted into the lower buffer tank and filled with electrode buffer. Electrophoresis was carried out with constant current of 15 mA in the stacking gel and 30 mA in the resolving gel until the marker dye approached 0.75 cm from the bottom of the gel. After the electrophoresis was complete, the distance of dye migration and the gel length were measured. The gel was stained with the silver stain by the method of Merril et al.[97] The distance of dye migration of the stained gel and its length were also measured.

9. Silver nitrate staining

Although the coomasie blue staining technique was found to be quite satisfactory, in some situations a more sensitive staining tecnique was required through which silver nitrate staining was selected as it could detect protein as little as 0.01 nanogram per square millimetre or 100 times more sensitive than coomasie blue stain. A photochemically derived staining procedure was described by Merril et al[97], as listed below.

The gel was fixed in 50% methanol and 12% glacial acetic acid for at least 20 min. Excess of sodium lauryl sulfate in the gel was removed by soaking it in 10% etanol and 5% acetic acid solution for about 10 min. The gel was then allowed to stand in 0.0034 M

potassium dichromate and 0.0032 N nitric acid solution for 5 min, washed at least four times in DDW and transferred to a 0.012 M silver nitrate solution for 30 min. The gel was rinsed rapidly with a large volume of image developer twice, allowing the gel to stand in the developer with gentle agitation until the staining pattern reached a desired intensity (about 3 min), the reaction was stopped by transferring the gel to 1% acetic acid solution. After 5 min, the gel was washed twice with DDW. The stained gel can be kept in DDW for several months. All reagents that were used for silver nitrate staining were prepared as described in Appendix 9.

After staining, the stained gels were photographed and estimated for molecular weight of components.

10. Determination of molecular weight of components by SDS-PAGE

The molecular weight of polypeptides was determined from a standard curve, plotted on a semilogarithmic scale by using a commercial protein standards.(Appendix 10)

Relative mobility of the unknown polypeptide was calculated by the following formula[94].

$$\text{Relative mobility} = \frac{\text{Distance of protein migration}}{\text{Gel length after staining}} \times \frac{\text{Gel length before staining}}{\text{Distance of dye migration}}$$

For accuracy and precision, the standard proteins were always electrophoresed simultaneously on the same gel with the unknown

specimens.

11. Optimization of protein concentration of sonic extract of *P. pseudomallei* for studying its pattern in silver stained SDS-PAGE

The optimum of protein concentration was determined by using sonic extract of *P. pseudomallei* NCTC 4845. The various concentration; 2,5 and 10 ug protein/lane were performed on silver stained SDS-PAGE.

12. Scanning and analysis of data

The stained SDS-polyacrylamide gels were soaked in a solution, containing 40% methanol and 5% glycerol (Appendix 11), at least 2 hrs, and then dried between two sheets of cellophane by using LKB 2003 Slab Gel Dryer. then the gels were directly scanned by LKB 2202 Ultrascan XL Laser Densitometer, which was connected with an LKB 2400 Gel Scan XL Soft Ware Package and IBM Compatible Computer.

The data on peak areas, heights, locations and % relative area were recorded by a densitometer scanning LKB 2220 Recording Integrator and comparison of the intensity of bands was made by grading the height of peak as follows

+++ = band present at height of peak > 0.8

++ = band present at height of peak > 0.6-0.8

+ = band present at height of peak < 0.45

W = band present at height of peak > 0.3

- = band not record in this position

The location of peak compared with the location of stnadard marker suggested the molecular weight of band.

13. Immunoblot procedure[85]

After an electrophoresis, the gels were soaked in transfer-buffer (Appendix 12.1) for 30 min and then electrobloted onto a nitrocellulose membranes (pore size 0.45 um) in a transblot apparatus, modified from the method of Towbin[85] as follows:

13.1 The blotting buffer (Towbin buffer) was poured into a Western transfer-chamber in which a piece of Nitrocellulose membrane, (NC), two pieces of fitter paper and two scotch-brite scouring pads were soaked to make them wet.

13.2 A sandwich was performed through the successive layers as shown in Fig 4. This sandwich was assembled quickly to avoid dehydration of the gel. Great care was taken to remove all air bubbles which would lead to the uneven transfer. It was then placed into the chamber between the electrodes with the nitrocellulose sheet towards the anode. Then the transferred buffer was added to fill the chamber.

13.3 Electrophoretic transfer was performed at 250 mA for 3 hrs and then followed by 50 mA for 18 hrs.

13.4 The NC membrane was removed from the chamber, tranferred into a plastic box containing 0.1% Tween 20 in phosphate buffer saline (PBS/T) (Appendix 12.3), rinsed and air dried. Then the strip was kept at 4 °C in plastic bag.

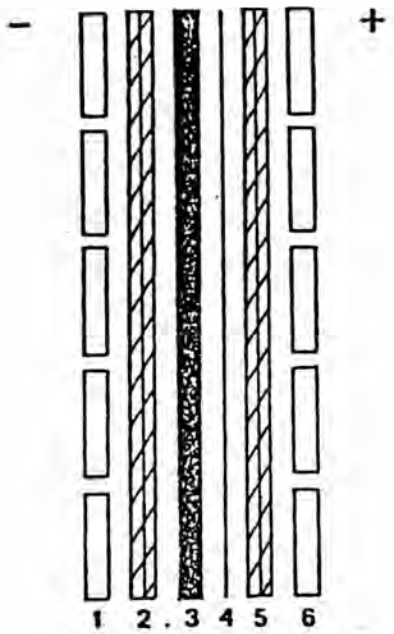

Fig 4 Diagram showing the assembly of electrophoretic transfer.

1. a supportative porus pad such as "Scotch Brite" scouring pad,
2. filter membrane soaked in Towbin buffer
3. the SDS-PAGE with the stacking gel removed.
4. a sheet of nitrocellulose cut to the size of the gel, prewet in Towbin buffer
5. an another filter paper soaked in Towbin buffer; and
6. another "Scotch Brite" scouring pad.

13.5 Visuallize for protein molecular weight markers on NC sheet by staining with India ink as described by Hancock[96].

13.6 Soak the NC sheet for immunostaining in 5% non fat dry milk (NFM) which was diluted in PBS/T for 3 hrs at 37 °C to saturated unused protein binding sites in order to minimize the background. Then rinse the NC sheet in PBS/T and air dried then kept at 4 °C in plastic bag for further staining.

13.7 Rinse the NC sheet in PBS/T and incubate for 3 hrs at 37 °C with either rabbit antiserum to *P. pseudomallei* Ig or normal rabbit Ig at an optimal determined in 5% NFM in PBS/T.

13.8 After incubation, wash the NC sheet for five times (10 min soaking time) in 100 ml PBS/T to remove excess unbound antiserum.

13.9 Incubate the NC sheet for 1 hr at 37 °C with peroxidase conjugated swine antirabbit Ig at an optimal dilution which was previously determined (1:50 in PBS/T).

13.10 Wash off the excess unbounded anti Ig with PBS/T for three times with shaking, (10 min soaking time) remove the buffer off.

13.11 Incubate the NC sheet with substrate solution at room temperature which stored in dark (Appendix 12.7) to reveal the polypeptides involved in antigen antibody reaction. The reaction was terminated when blue color was visible (about 30 min) and then washed with DDW. Dry the NC sheet between filter papers and store in dark to prevent fadding of the patterns. It was photographed as early as possible for the record.

14. India ink staining

After immunoblottin, the molecular weight markers on NC could be visuallized by India ink staining method as described by Hancock et al[98]. It could be detected as sensitive as 80 ng of protein. The NC strip was washed four times for 10 min each in 200 ml PBS/T at 37 °C (Appendix 13.1), in a shaker incubator in order to remove any adherent polyacrylamide gel particles and residual SDS which may interfere with the staining. The NC strip was then stained with 200 ml of the solution of Pelikan fount india drawing ink which was diluted 1:100 in PBS/T. Usually the stained bands appeared in 1 hr. Finally it was rinsed with DDW 5 min and then air dried.

15. Optimization of antigen concentration and antibody dilution used in immunoblotting technique

To optimize the antigen concentration, the various concentrations 2,5 and 10 ug protein per lane of sonic extract of *P. pseudomallei* NCTC 4845 were electrophoresed in SDS-PAGE and transferred onto NC after which they were incubated with homologous rabbit antiserum immunoglobulin at the dilution of 1:100.

To optimize the antibody dilution, the homologous antisera dilutions of 1:50, 1:100 and 1:200 were used to react with the antigen which obtained from 5 ug protein per lane SDS-PAGE and transferred onto NC strip. These optimization were followed by the method as described before.