CHAPTER IV

RESULTS

1. Characteristics of *P. pseudomallei*

Thirty five strains were verified as *P. pseudomallei* by the method of Gilardi[31]. The characteristics were shown in Table 6

2. Protein content of sonic extract of *P. pseudomallei* and other bacteria

The protein concentrations of sonic extract of all bacteria (in Table 4) was determined by modified Lowry assay[87], were shown in Table 7.

3. Preperation and isolation of normal rabbit immunoglobulin and rabbit antiserum (gamma globulin) against *P. pseudomallei.*

Rabbit serum was obtained before subcutaneous inoculation of sonic extract of *P. pseudomallei* NCTC 4845. The schedule of bleedings and immunizations with these antigens was shown in Table 8. The antibody level of normal serum against *P. pseudomallei* was less than 1:10 by indirect haemagglutination.[88] The titer of the immune-sera were ranging from 1:40 to 1:5,120. The normal rabbit serum and the immune sera with titer of 1:5,120 were further purified for immunoglobulin by salting-out method[89]. After which the titer of

Table 6 Characteristics of 35 strains of *P. pseudomallei* and reference strain (NCTC 4845)

Test Performed	NCTC 4845 Sign	*P. pseudomallei* Sign	*P. pseudomallei* %(+)
Morphology	mrs(a)	mrs(a)	100
Flagella	p>2(b)	p>2(b)	100
Motility	+	+	100
Action on blood	cc H	V(c)	77(27)
Fermentative or Oxidative	O	O	100
Carbohydrate base	OF	OF	
Acid from Glucose	+	+	100
Xylose	+	+	97(34)
Mannitol	+	+	100
Lactose	+	+	100
Sucrose	+	V	88(30)
Maltose	+	+	100
Mannose	+	+	100
Galactose	+	+	94(33)
Fructose	+	+	100
Rhamnose	-	V	60(21)
10% Lactose	+	+	100
Catalase	+	+	100
Oxidase	+	+	100
TSI slant acid (2 days)	+	+	100
TSI butt acid	-	-	100
H_2S (TSI butt)	-	-	100
H_2S (pb ac paper)	-	-	100
ONPG	-	-	8(3)
Simmon citrate	+	+	100
Urea Christensen's	-	V	57(20)
Nitrate reduction	+	+	100
Gas from nitrate	+	+(d)	100
Indol	-	-	100
MR	-	-	100
VP	-	-	100
2-ketogluconate	-	V	71(25)
Malonate	-	V	77(27)
Phenylalanine deaminase	-	-	100
Esculin hydrolysis	+	V	82(22)
Tween 80 hydrolysis	+	V	49(17)
Starch hydrolysis	+	+	100
Gelatin hydrolysis(e)	+	+	100
Lysine decarboxylase	-	-	100
Arginine dihydrolase	+	+	100
Ornithine decarboxylase	-	-	100
Growth on Mac Conkey	+	+	100
SS	-	V	20(7)
Nutrient broth 8.5% NaCl	-	-	100
Growth at 42 °C	+	+	100

Note : (a) = middle stright rod, bipolar staining
(b) = more than 2 polar flagella
(c) = greening of blood, usually accompained by lysis; growth
(d) = the volume of gas may be small
(e) = within 14 days
+ = positive reaction (90% or more than strains tested were positive) within 48 hr, except with gelatin that within 14 days.
- = negative reaction (10% or less strains tested were positive)
V = variable (11-89% tested positive)

Table 7 Protein concentration of sonic-extract of 45 strain as determined by modified Lowry method

strain number	Sonic-extract of bacterial strains Laboratory designation	Protein concentration (mg/ml)
1.	*P.pseudomallei* CPp 01/85	3.75
2.	*P.pseudomallei* CPp 02/85	4.25
3.	*P.pseudomallei* CPp 02/86	4.50
4.	*P.pseudomallei* CPp 03/86	4.25
5.	*P.pseudomallei* CPp 04/86	4.55
6.	*P.pseudomallei* CPp 05/86	3.85
7.	*P.pseudomallei* CPp 06/86	3.30
8.	*P.pseudomallei* CPp 01/87	3.20
9.	*P.pseudomallei* CPp 02/87	3.65
10.	*P.pseudomallei* CPp 05/87	3.25
11.	*P.pseudomallei* CPp 06/87	3.35
12.	*P.pseudomallei* CPp 07/87	4.35
13.	*P.pseudomallei* CPp 01/88	3.85
14.	*P.pseudomallei* CPp 03/88	3.40
15.	*P.pseudomallei* CPp 04/88	3.25
16.	*P.pseudomallei* CPp 05/88	2.50
17.	*P.pseudomallei* CPp 06/88	4.00
18.	*P.pseudomallei* CPp 07/88	3.50
19.	*P.pseudomallei* SPp 01/84	3.85
20.	*P.pseudomallei* SPp 01/87	2.20
21.	*P.pseudomallei* SPp 02/87	3.40
22.	*P.pseudomallei* SPp 03/87	3.20
23.	*P.pseudomallei* SPp 04/87	3.57
24.	*P.pseudomallei* SPp 05/87	3.20
25.	*P.pseudomallei* SPp 06/87	3.30
26.	*P.pseudomallei* CMPp 01/88	4.78
27.	*P.pseudomallei* CMPp 01/87	3.40
28.	*P.pseudomallei* CMPp 02/87	3.88
29.	*P.pseudomallei* CMPp 03/87	3.42
30.	*P.pseudomallei* KPp 01/87	3.20
31.	*P.pseudomallei* KPp 02/87	3.17
32.	*P.pseudomallei* KPp 01/88	4.35
33.	*P.pseudomallei* DMSD 0732	3.05
34.	*P.pseudomallei* DMSD 0734	3.48
35.	*P.pseudomallei* Cow Pp01/88	4.35
36.	*P.pseudomallei* NCTC 4845	4.15
37.	*P.aeruginosa* ATCC 27853	4.35
38.	*P.cepacia* JCM 5510	3.05
39.	*P.stutzeri* JCM 5965	3.42
40.	*P.putida* JCM 8160	3.50
41.	*P.maltophila* JCM 3801	4.30
42.	*V.cholerae* 569 B	4.50
43.	*S.typhi* NCTC 781	2.20
44.	*E.coli* ATCC 25822	2.85
45.	*S.aureus* ATCC 25923	3.40

Table 8 Schedule for immunization and the level of indirect haemagglutination titers at various vaccination technique

On Day	Subcutaneous injection (mg of protein of sonic extract of *P.pseudomallei* NCTC 4845)	IHA
0	-	< 1:10
1	1.25	< 1:10
7	-	1:40
14	0.625	1:640
21	0.625	1:1280
28	-	1:5120
30	-	1:5120
35	-	1:5120

immunoglobulin obtained was determined; the normal rabbit immunoglobulin was at < 1:10, and immune serum immunoglobulin was 1:10,240 by indirect haemaglutination test.

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

It was shown in Fig 5 that the 5 ug of protein per lane of sonic extract appeared to be best resolution. It was, therefore, the concentration that was used in all of the experiments in SDS-PAGE.

5. Patterns of sonic extract of all *P. pseudomallei* in SDS-PAGE

The component profile of sonic extract of *P. pseudomallei* in the total of 36 strains were studied by SDS-PAGE. The protein concentration used in each of these strains was 5 ug protein per lane.

The results in each strain which produced similar pattern in which they were containing at least 40-50 discreted visualizable bands having molecular weights between 12.0-190.0 Kd (Fig 6). There were nine major intensive bands with molecular weight 13.9-57.1 Kd (13.9, 15.0, 18.6, 20.7, 28.5, 31.0, 34.2, 48.8, and 57.1 kd)presented in all strains It was noted that high molecular weight region (greater than 57.1 Kd) of all strains had almost identical pattern. However, variation of the pattern in SDS-PAGE profile was observed in the region within molecular weight range 12.6-56.0 Kd. Differences were found in the presence and absence of particular bands as well as in the varying intensity of these bands which was detectable by densitometer. The degree of intensity of

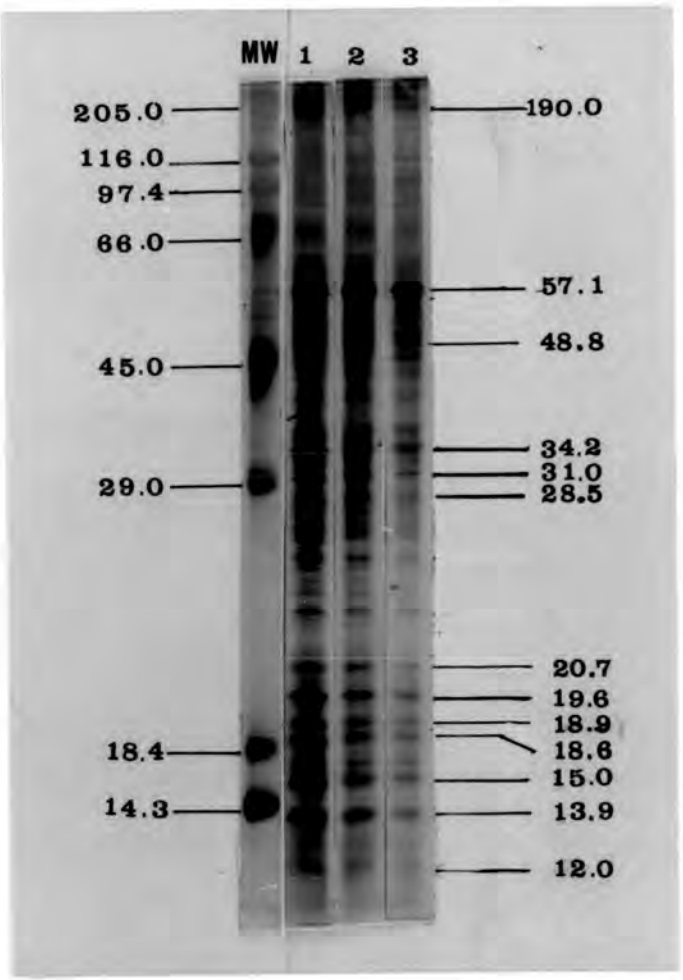


Fig 5. Silver stained SDS-PAGE profile of sonic-extract of *P. pseudomallei* NCTC 4845. The extract at concentrations of 10, 5, and 2 ug protein per lane were shown in lane 1, 2, 3 respectively. The molecular weight markers (MW) from top to bottom were myosin (205.0), beta-galactosidase (116.0), phosphorylase B (97.0), albumin bovine (66.0), egg albumin (45.0), carbonic anhydrase (29.0), beta-galactoglobulin (18.4) and lysozyme (14.3). The relative MW.(Kd) are shown on the right hand side of the figure.

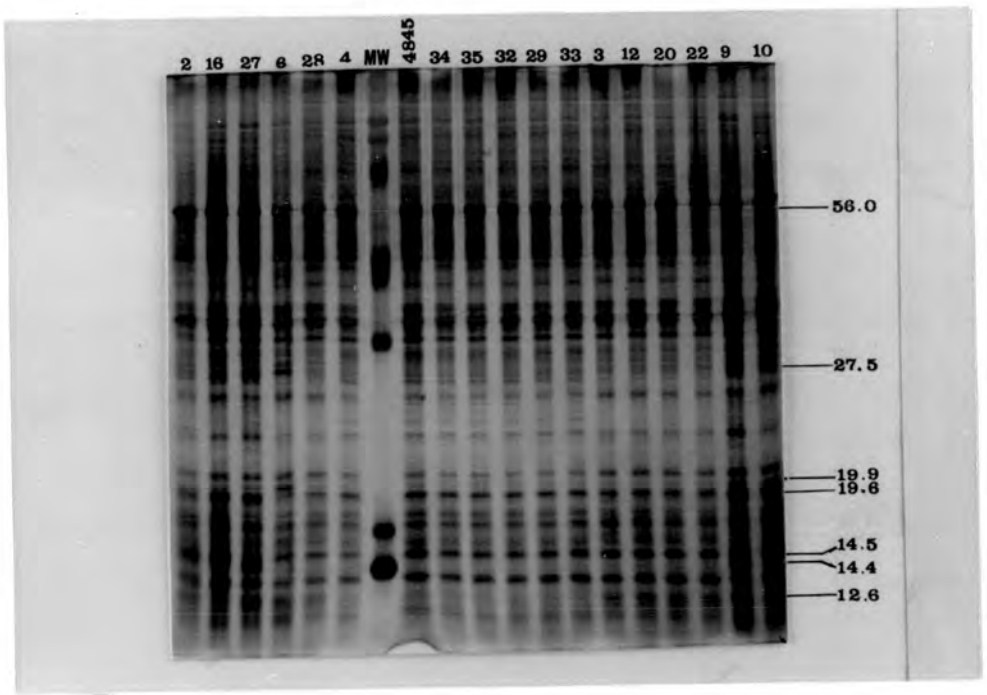


Fig 6 Silver stained SDS-PAGE profile of sonic extract of *P. psuedomallei*. The label number on the top is strain number which were described in Table 4

bands at the molecular weights of 56.0, 27.5, 19.9, 19.6, 14.5, 14.4 and 12.6 Kd was used for the criteria in the typing of 36 strains of *P.pseudomallei*,and they were divided into 6 types as shown in Table 9.

The typical patten of six SDS-PAGE Types were illustrated in Fig 7 and the distribution of those types were shown in the Table 10 and Fig 8-14.

The percentage of 32 clinical isolates of *P. pseudomallei* were shown in Fig 15.

6. Immunoblot analysis of sonic extract of *P. pseudomallei*.

The antigenic profile among 36 strains of *P. pseudomallei* was determined by immunoblotting technique[95]. The antiserum used in this study rabbit anti-*P. pseudomallei* NCTC 4845 immunoglobulin as described in material and methods.

After an electrotransferred of the bands from SDS-PAGE onto a nitrocellulose, proteins which were not completely transferred in the gel were detected by silver staining (Fig 16 A). It was appeared that trace amount of remaining protein in high molecular weight region in the gel was demonstrated by the silver stain (Fig 17).

In addition, to assure that the bands were transferred onto the nitrocellulose strip, the NC was stained with 0.01% India ink. The stained NC strip revealed a pattern of bands identical to that of silver stained SDS-PAGE before electrotransferring (Fig 16 B).

Table 8 The patterns of the significant bands in each SDS-PAGE types of *P. pseudomallei*

SDS-PAGE type	presence of bands* of MW (Kd)						
	56.0	27.5	19.9	19.6	14.5	14.4	12.6
I	++	+++	-	++	-	-	W
II	+	++	-	++	+	-	++
III	W	++	+++	W	-	-	+++
IV	W	+++	W	++	-	+++	+++
V	W	+++	-	++	++	-	++
VI	W	+	-	++	-	-	++

* By mean of densitometer scanning

+++ = Band present at heigh of peak > 0.8

++ = Band present at heigh of peak 0.6-0.8

+ = Band present at heigh of peak <0.45

W = Band present at heigh of peak <0.30

\- = Band not record in this position

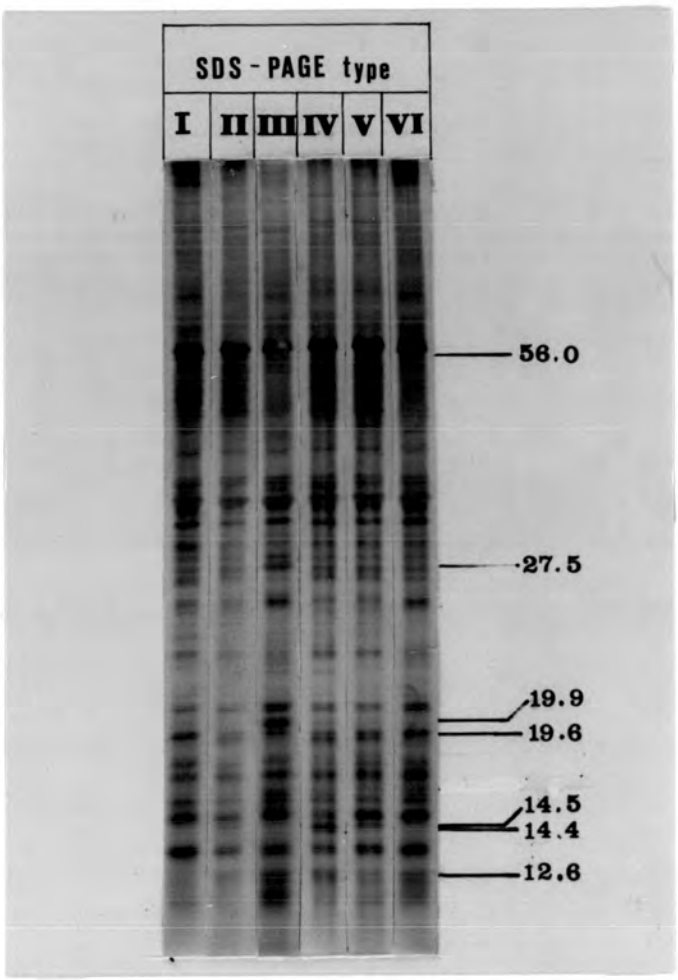


Fig 7 Silver stained SDS-PAGE profile of sonic extract of *P. pseudomallei* Type I-VI

Table 10 Summary classification of 36 strains of *P. pseudomallei* in to SDS-PAGE types

SDS-PAGE Type					
I	II	III	IV	V	VI
1*	2	6	9	8	3
4	5		23	16	11
7	10		27	24	12
15	13				14
18	17				20
25	19				21
26					22
29					28
32					30
33					31
34					
35					
36					

* strains number

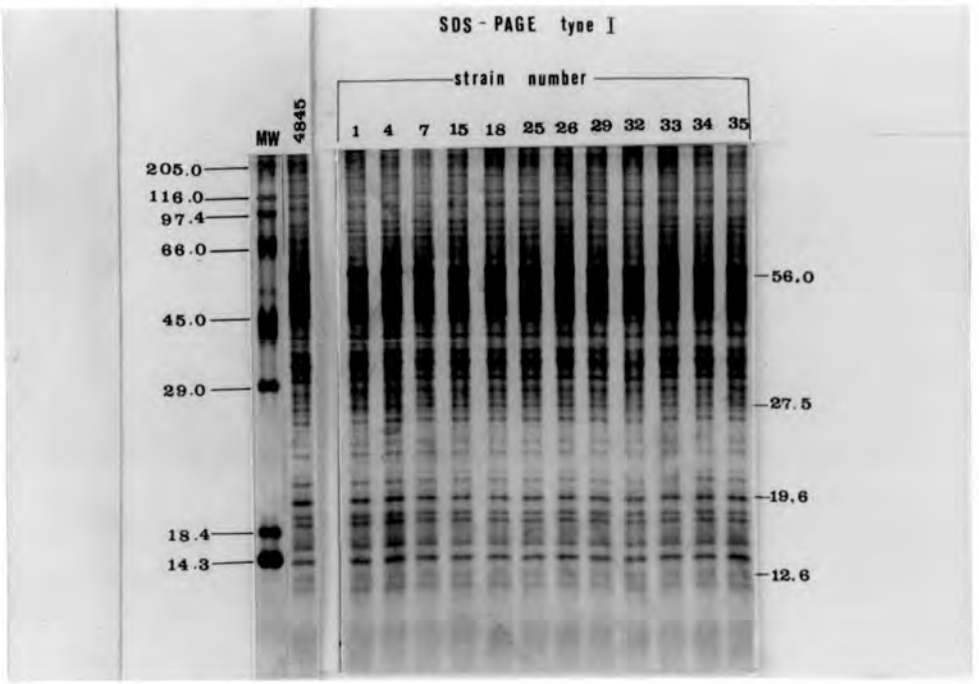


Fig 8 SDS-PAGE of sonic extract of *P. pseudomallei* NCTC 4845 and Type I of *P. pseudomallei* stained with silver. The lane numbers are refered to strain numbers from Table 4. The relative molecular weights (Kd) are illustrated on the right-hand side of the figure.

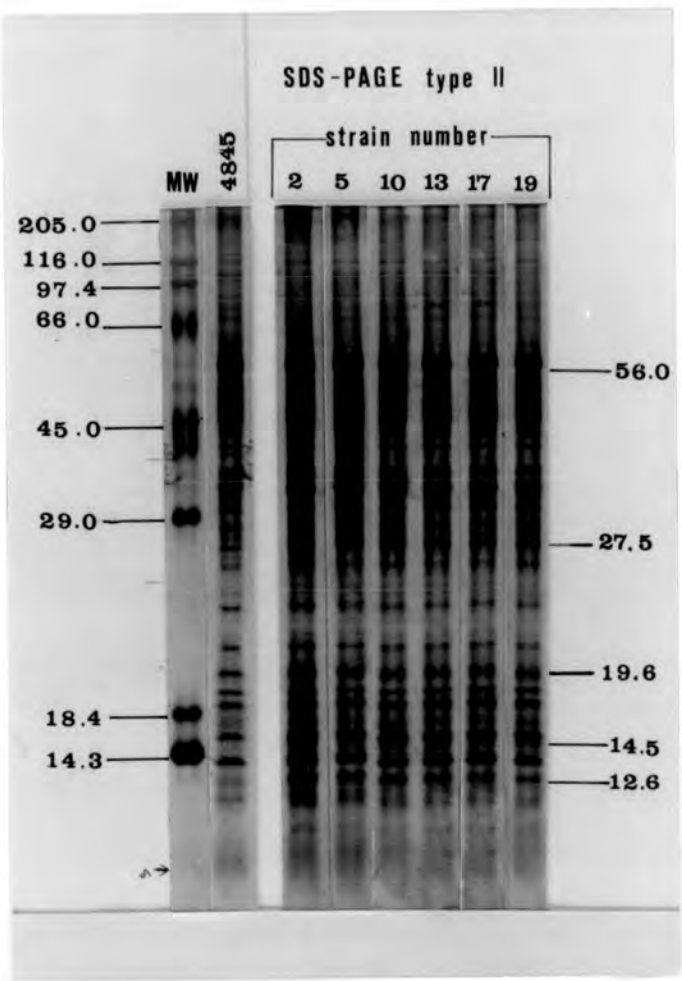


Fig 8 SDS-PAGE of sonic extract of *P. pseudomallei* NCTC 4845 and Type II of *P. pseudomallei* stained with silver. The lane numbers are refered to strain numbers from Table 4.

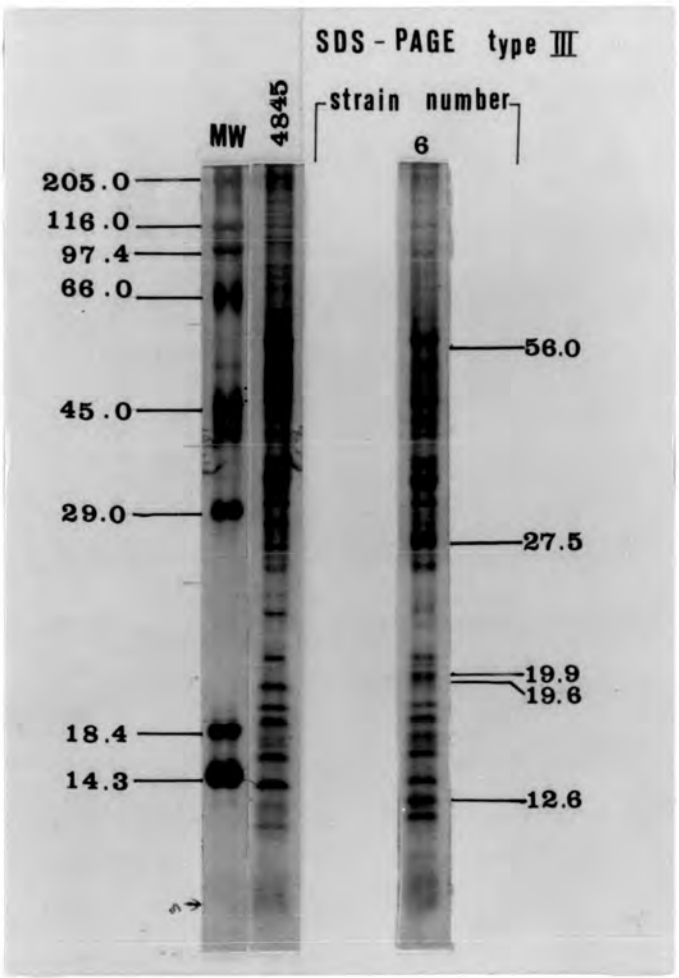


Fig 10 SDS-PAGE of sonic extract of *P. pseudomallei* NCTC 4845 and Type III of *P. pseudomallei* stained with silver. The lane numbers are refered to strain numbers from Table 4.

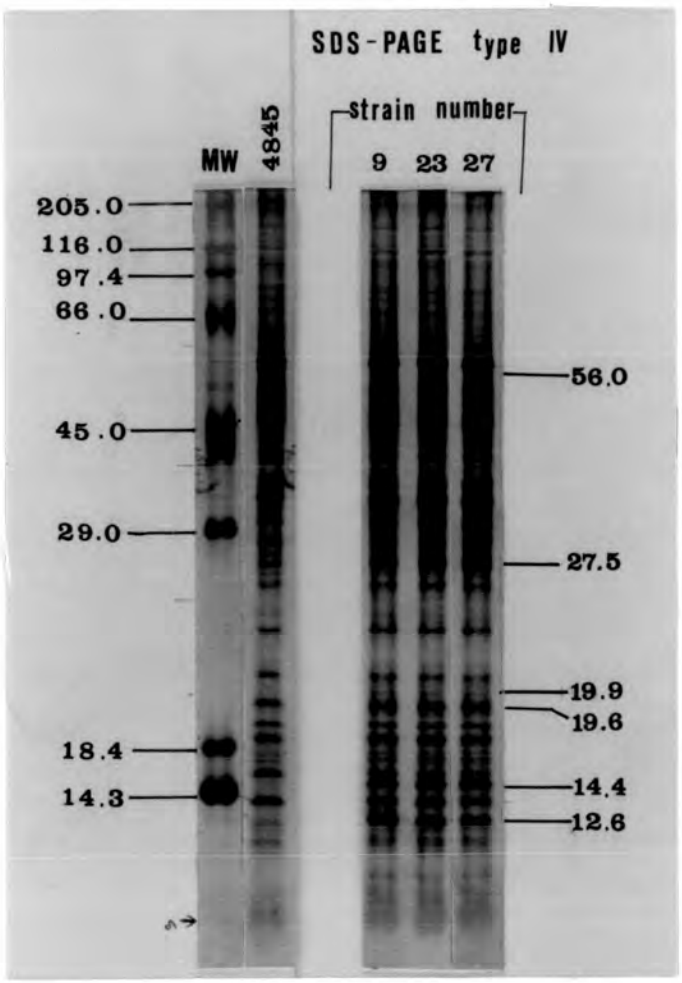


Fig 11 SDS-PAGE of sonic extract of *P. pseudomallei* NCTC 4845 and Type IV of *P. pseudomallei* stained with silver. The lane numbers are refered to strain numbers from Table 4.

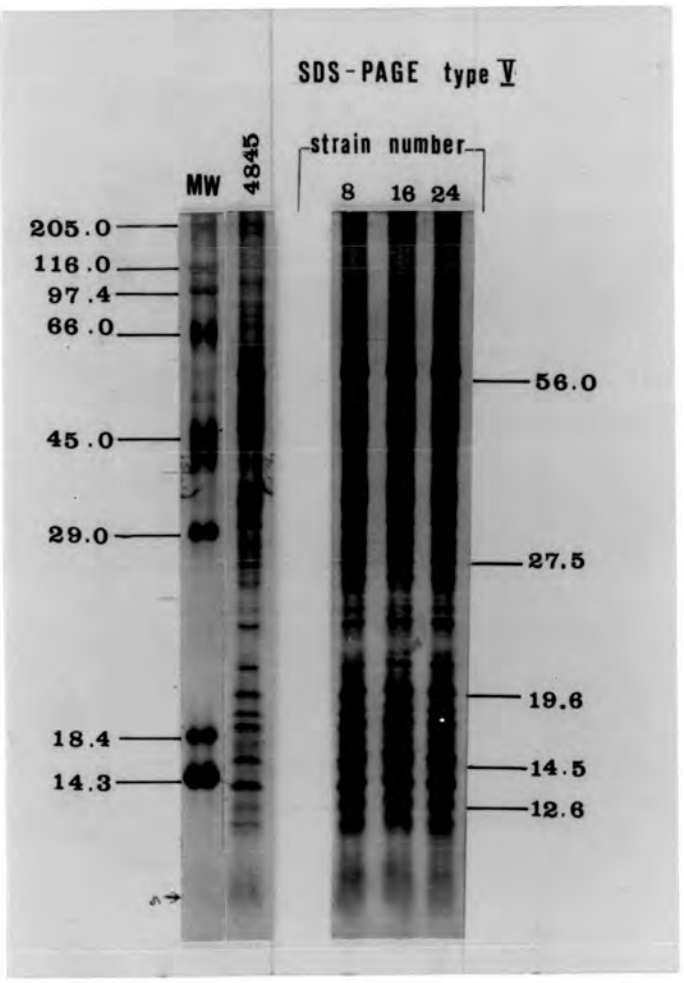


Fig 12 SDS-PAGE of sonic extract of *P. pseudomallei* NCTC 4845 and Type V of *P. pseudomallei* stained with silver. The lane numbers are refered to strain numbers from Table 4.

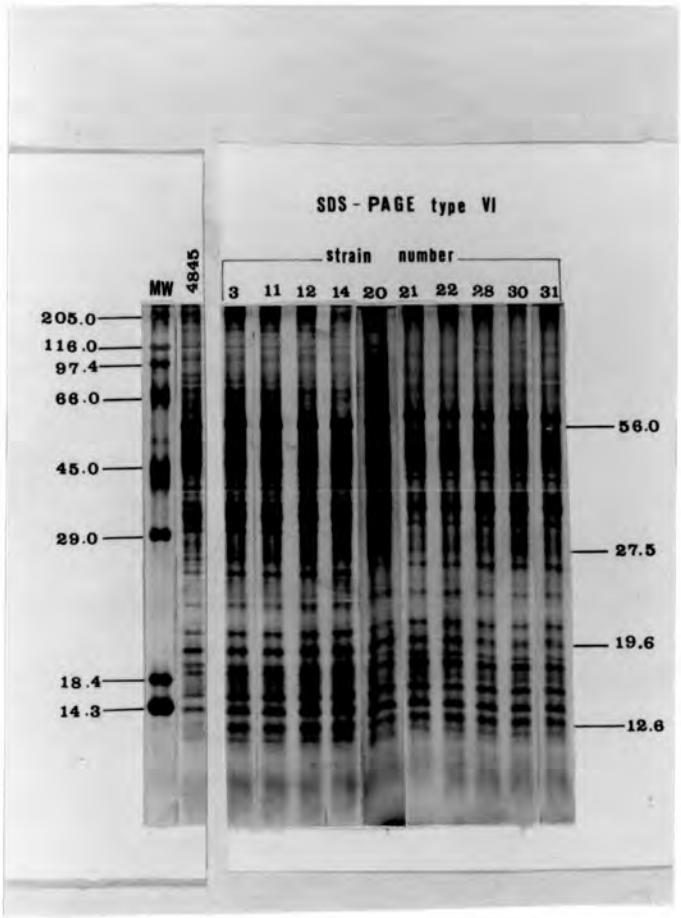


Fig 13 SDS-PAGE of sonic extract of *P. pseudomallei* NCTC 4845 and Type VI of *P. pseudomallei* stained with silver. The lane numbers are refered to strain numbers from Table 4.

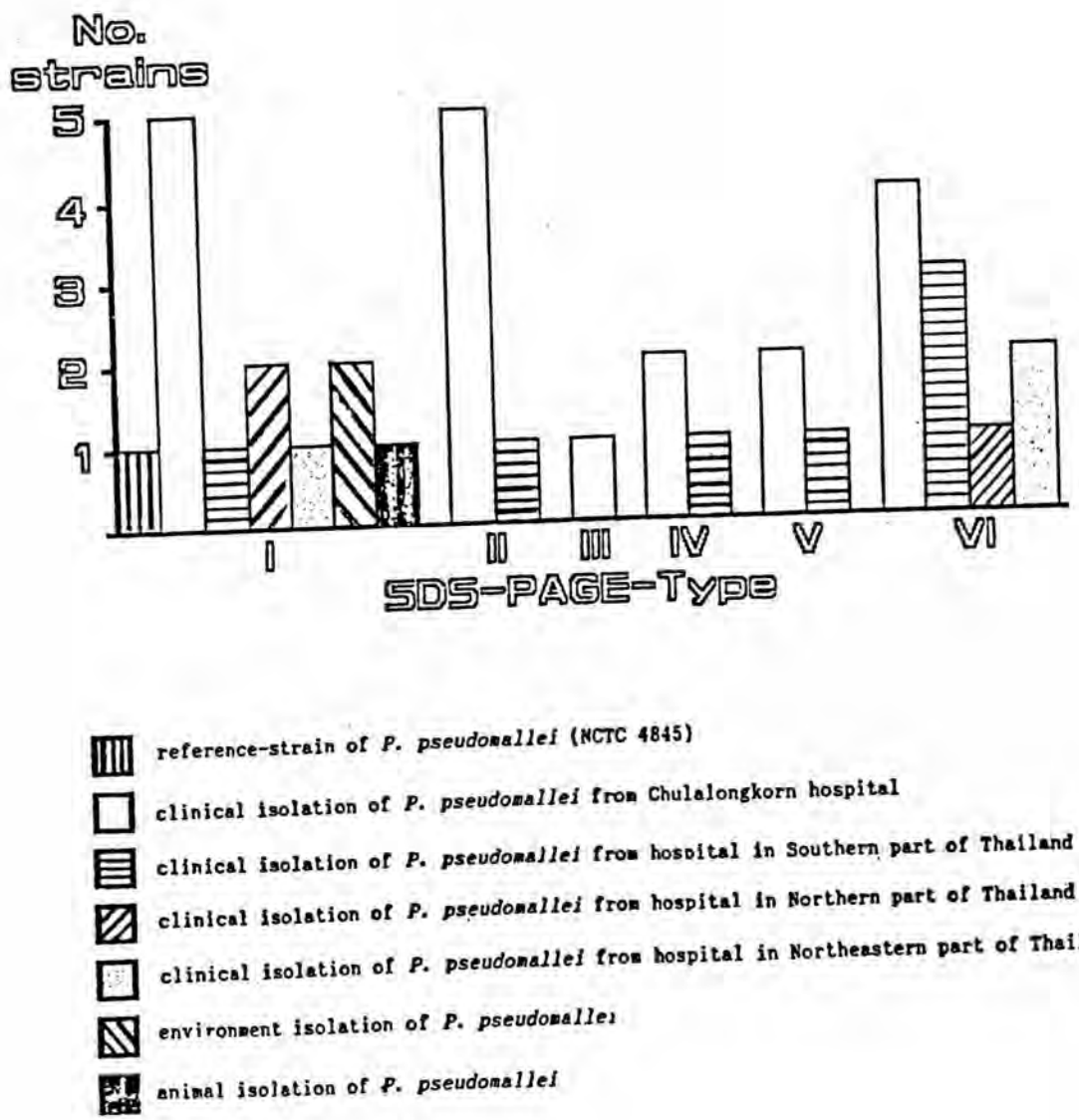


Fig 14 Distribution of 36 strains of *P. pseudomallei* in each SDS-PAGE Type.

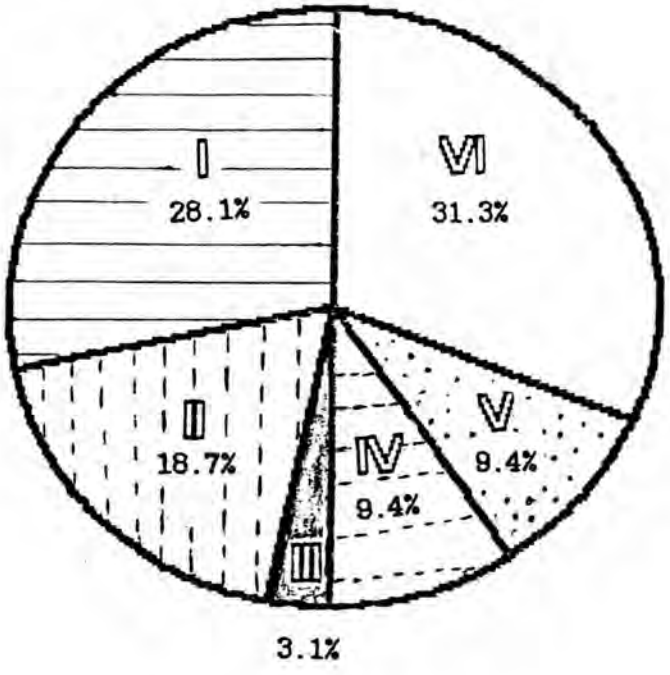


Fig 15 The percentage of 32 clinical isolates in each SDS-PAGE Type

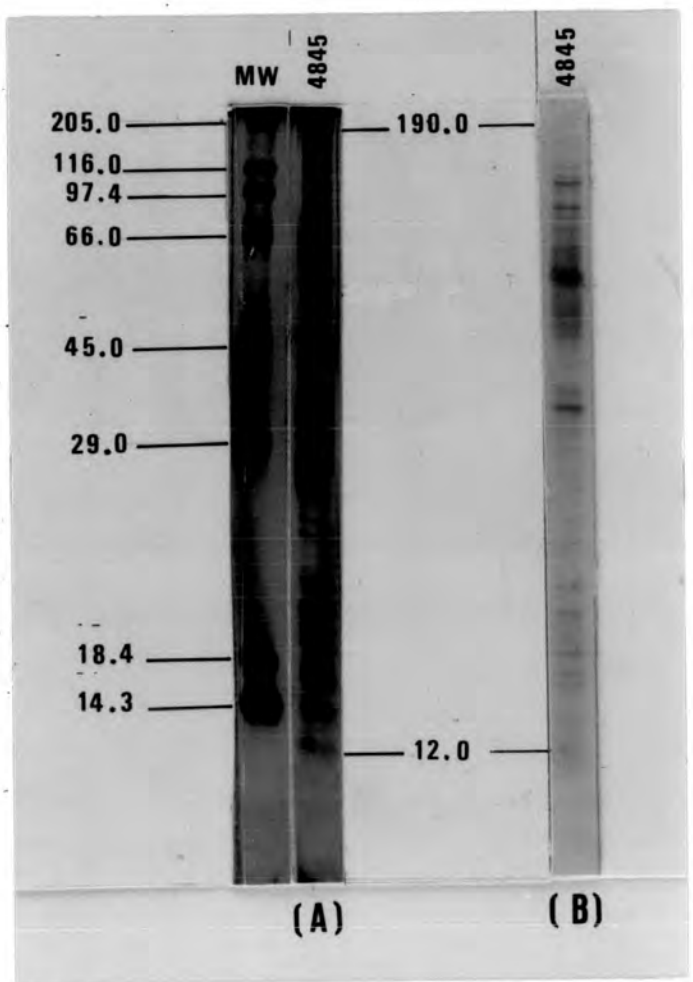


Fig 16 SDS-PAGE analysis of sonic extract of *P.pseudomallei* NCTC 4845, 5 ug protein per lane (A) and molecular weight markers stained with silver. The transferred bands onto the nitrocellulose membrane stained with India ink are illustrated on the right-hand side (B)

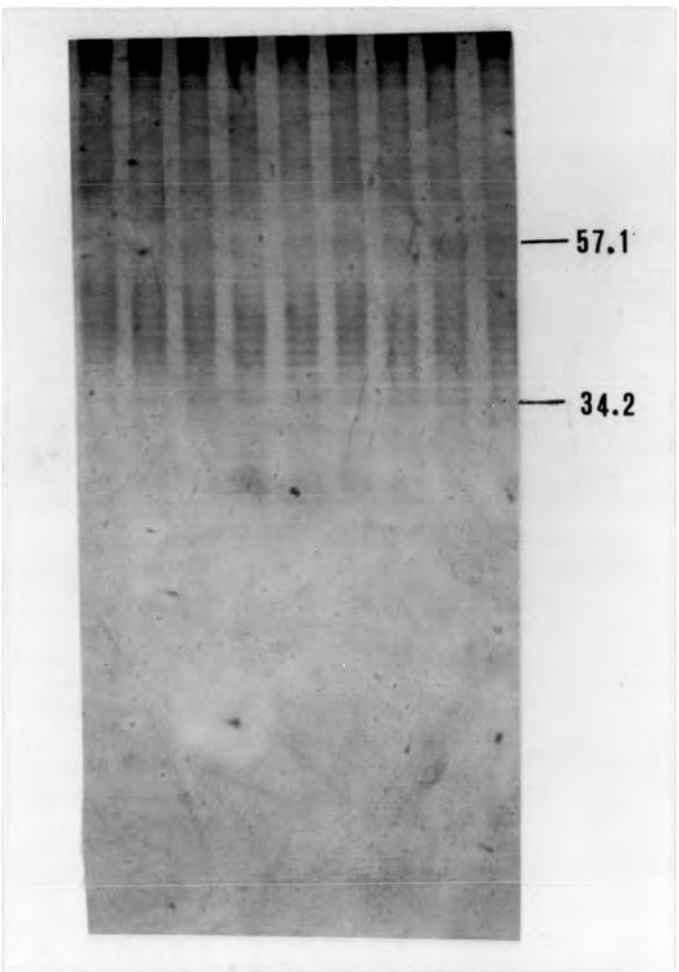


Fig 17 Silver stained of SDS-PAGE of sonic extract of *P. pseudomallei* after transferring onto nitrocellulose membrane.

6.1 Optimization of antigen concentration and antibody dilution used in an immunoblotting technique

6.1.1 Optimization of antigen concentration

The result in Fig 18 A showed that the suitable concentration of protein appeared to be 5 ug protein per lane and therefore this concentration of the protein was used through out the immunoblotting experiment

6.1.2 Optimization of antibody titer

The result in Fig 18 B showed the good resolution of discreted band by antibody dilution of 1:100. There for this dilution of antiserum was used through out the immunoblotting experiment.

6.2 Immunoblot analysis of *P. pseudomallei* NCTC 4845

The immunoblotting of *P. pseudomallei* NCTC 4845 reacted with homologous antiserum detected multiple antigenic bands with molecular weight of 12.0-140.0 Kd as shown in Fig 19. There were 18 major antigenic bands with molecular weight in the region of 18.6-115.0 Kd (18.6, 20.7, 26.4, 27.2, 34.2, 37.0, 43.0, 44.6, 46.5, 48.8, 49.1, 52.5, 56.0, 57.1, 67.0, 73.0, 79.0 and 107.0 Kd).

Patterns of immunoblotting of 35 strains which reacted with antiserum against *P. pseudomallei* NCTC 4845 were examined by direct visualization. The antigenic pattern of all strains were similar but not identical. The variations in the present and absent of bands at molecular weight 56.0, 15.0 and 14.4 were noted, and they

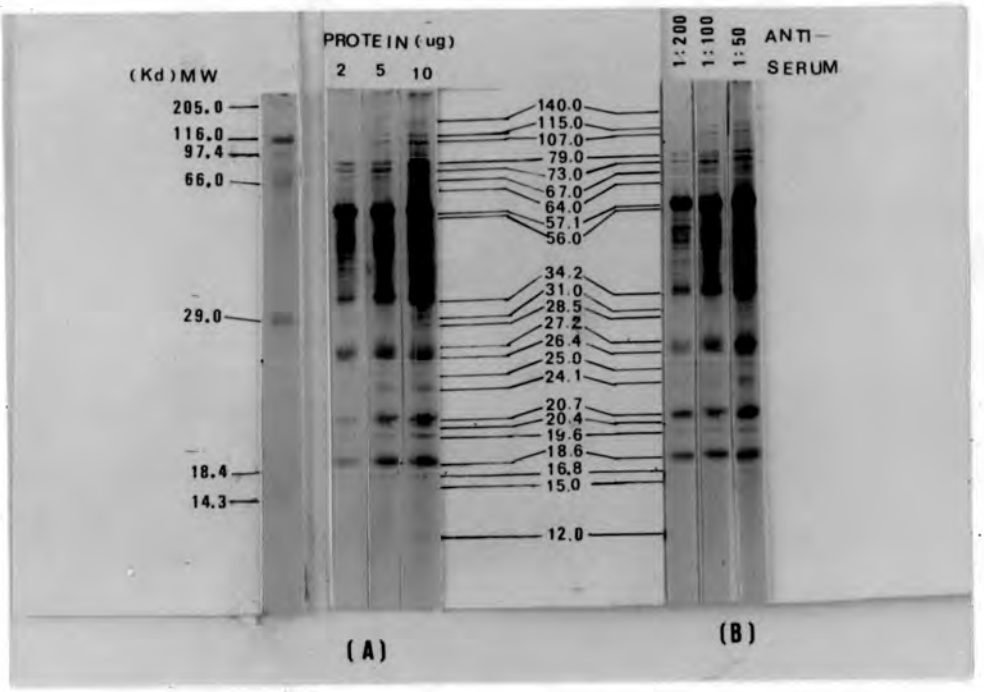


Fig 18. Immunoblot analysis of sonic extract of *P. pseudomallei* reference strain NCTC 4845, NC strips transferred from SDS-PAGE containing concentrations 2,5,10 ug protein per lane, were reacted with homologous rabbit antiserum diluted 1:100.(A) The lanes in the right-hand side are that of 5 ug protein per lane were reacted with homologous rabbit antiserum in various dilutions of 1:50, 1:100 and 1:200, followed by peroxidase conjugated swine antirabbit immunoglobulin and 4 chloro-1-naphthol substrate (B).

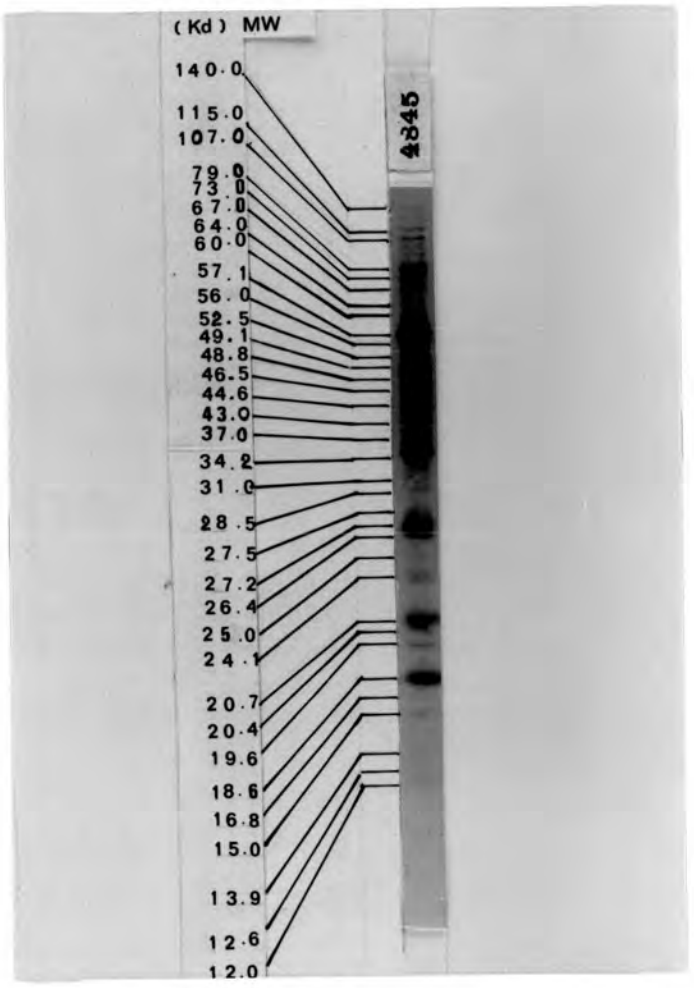


Fig 19 Immunoblot analysis of sonic extract of *P. pseudomallei* NCTC 4845 (5 ug protein per lane) reacted with homologous antiserum dilution 1:100

were used as criteria for deviding of the 35 isolates into 3 group, A, B, and C respectively. The pattern of difference in their antigenic bands was shown in Table 11 and Fig 20. Nineteen of the orginal 35 isolated strains and *P. pseudomallei* NCTC 4845 belonged to group A as shown in Fig 21, three isolated strains belonged to group B as shown in Fig 22, and the others belonged to group C as shown in Fig 23.

The distribution of 36 strains in each group was shown in Table 12 and Fig 24. Immunoblot classfication, group A, B and C of 32 clinical isolated strains have percentages of 46.8, 9.4 and 43.7 respectively as shown in Fig 25.

7. Immunoblot analysis of sonic extract of *P. pseudomallei* and other bacteria.

As expected, *P. pseudomallei* and other sero-cross reacting bacteria, *P. aeruginosa* ATCC 27853, *P. cepacia* JCM 5510, *P. stutzeri* JCM 5965, *P. putida* JCM 6160, *P. maltophila* JCM 3801, *V. cholerae* 569B, *S. typhi* NCTC 781, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 shared some common antigenic component by an immunoblot analysis. The profile of these bacteria in SDS-PAGE before electrotransferring was shown in Fig 26.

Immunoblotting strips reacted with normal rabbit sera showed antibody activity to some components of *P. pseudomallei* NCTC 4845 and other bacteria (as listed previously) at molecular weight of 57.1 and 59.0 Kd (Fig 27).

Table 11 Summary of the differences of bands in each pattern of 36 strains of *P.pseudomallei* by immunoblot technique that react with rabbit antiserum against *P.pseudomallei* NCTC 4845

Group	Total number of strain in each group	presence of bands MW (Kd)		
		58.0	15.0	14.4
A	19	+	+	-
B	3	+	-	+
C	14	-	+	-
Total	36			

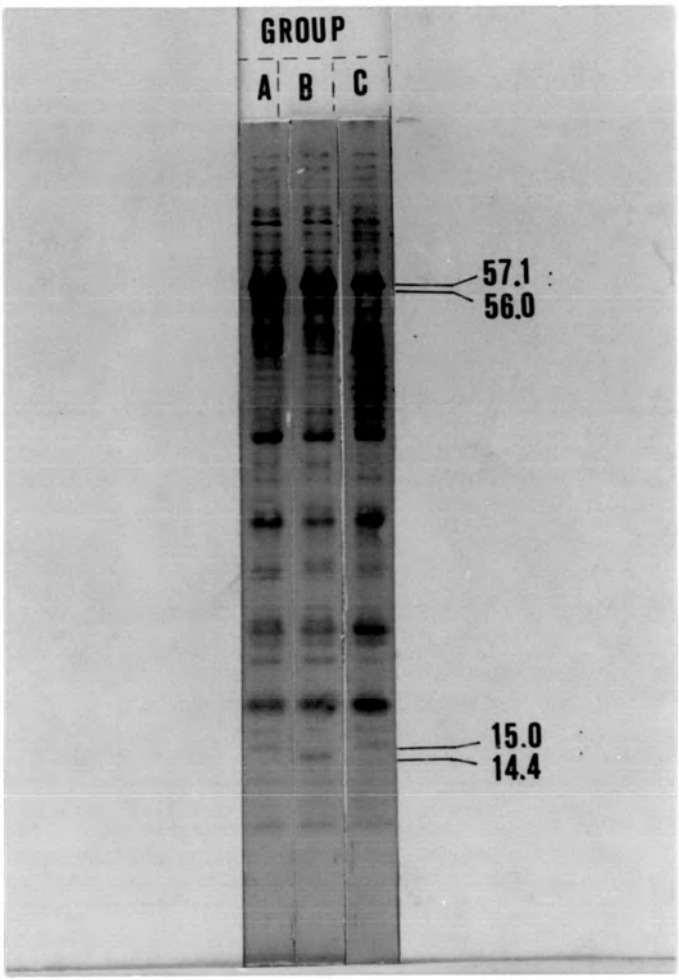


Fig 20 The antigenic patterns of Immunoblot analysis of *P. pseudomallei*

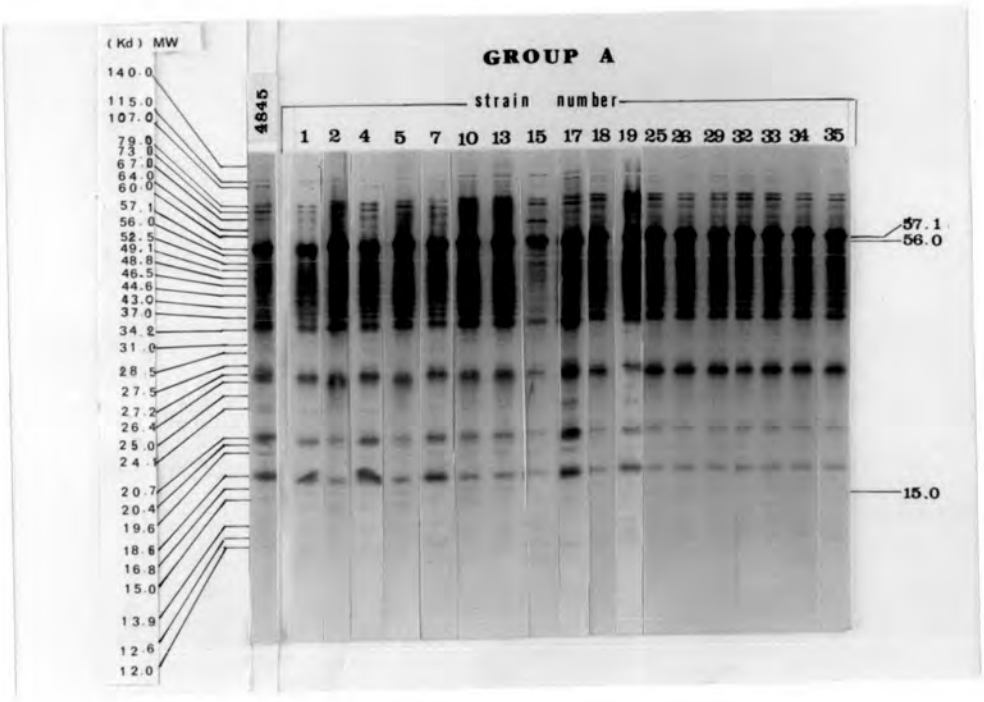


Fig 21 Immunoblot analysis of *P. pseudomallei* group A

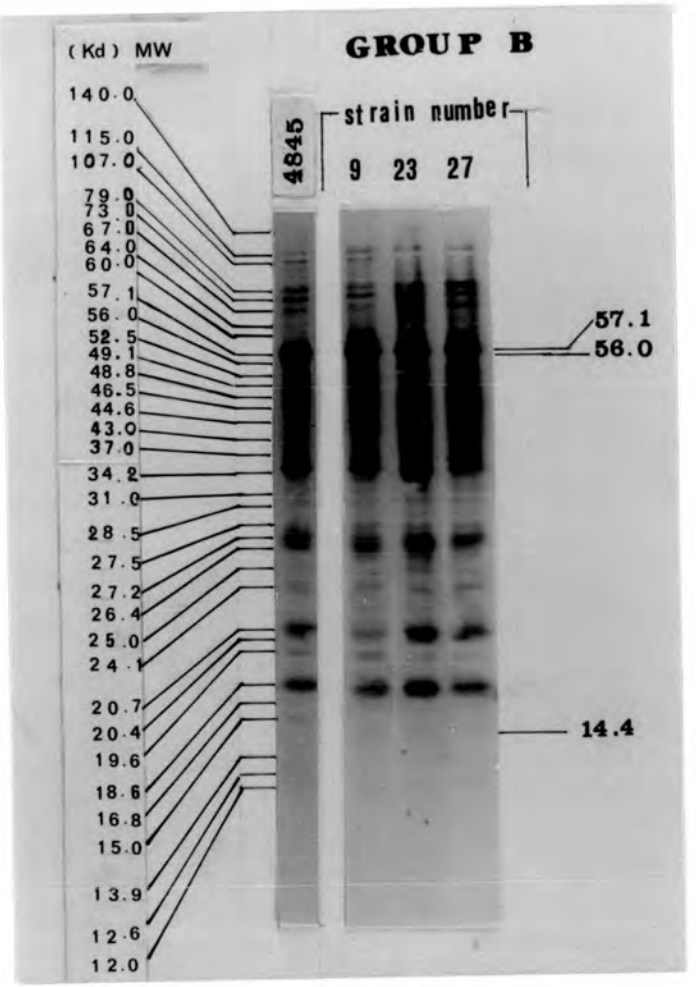


Fig 22 Immunoblot analysis of *P. pseudomallei* group B

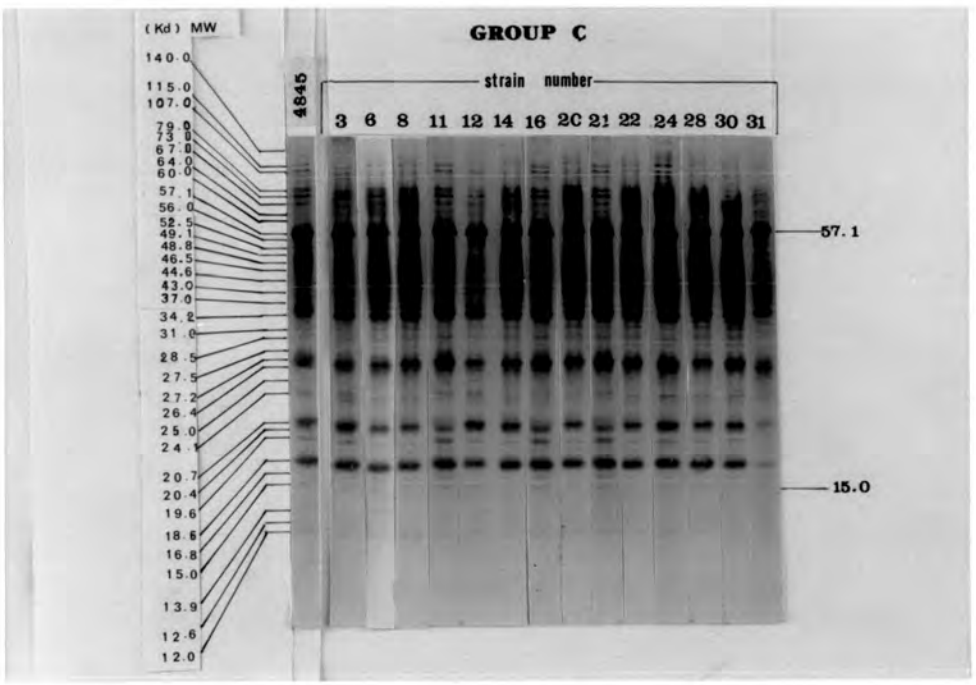
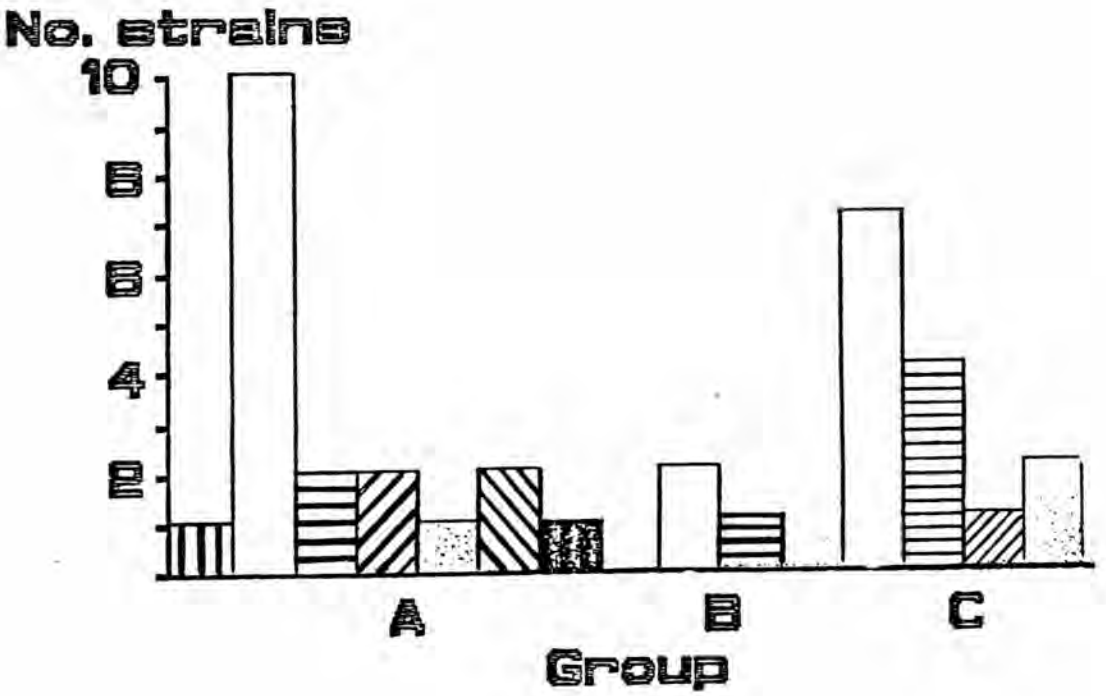


Fig 23 Immunoblot analysis of *P. pseudomallei* group C

Table 12 Summary classification of 36 strains of *P. pseudomallei* in each group by immunoblot technique

GROUP		
A	B	C
1*	9	3
2	23	6
4	27	8
5		11
7		12
10		14
13		16
15		20
17		21
18		22
19		24
25		28
26		30
29		31
32		
33		
34		
35		
36		

* strains number



reference-strain of *P. pseudomallei* (NCTC 4845)

clinical isolation of *P. pseudomallei* from Chulalongkorn hospital

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

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

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

environment isolation of *P. pseudomallei*

animal isolation of *P. pseudomallei*

Fig 24 Distribution of 36 strains of *P. pseudomallei* in each group by Immunoblot

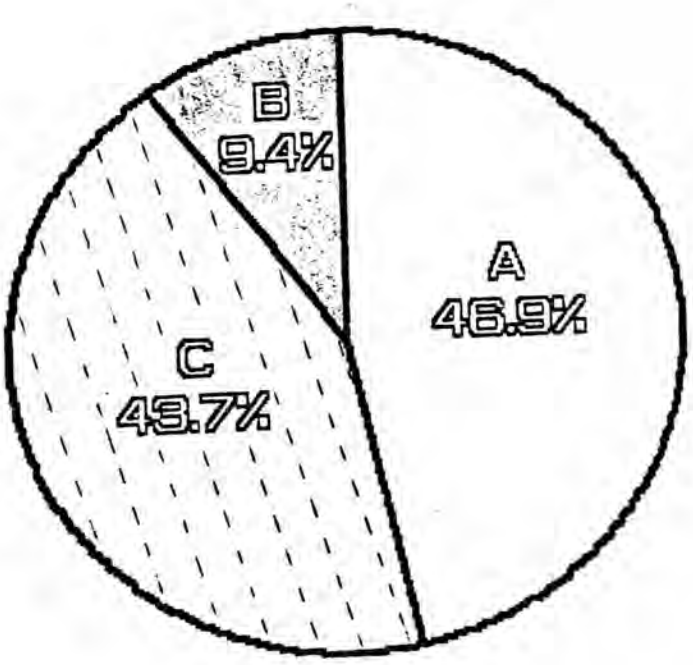


Fig 25 The percentage of 32 clinical isolates in each Immunoblot group

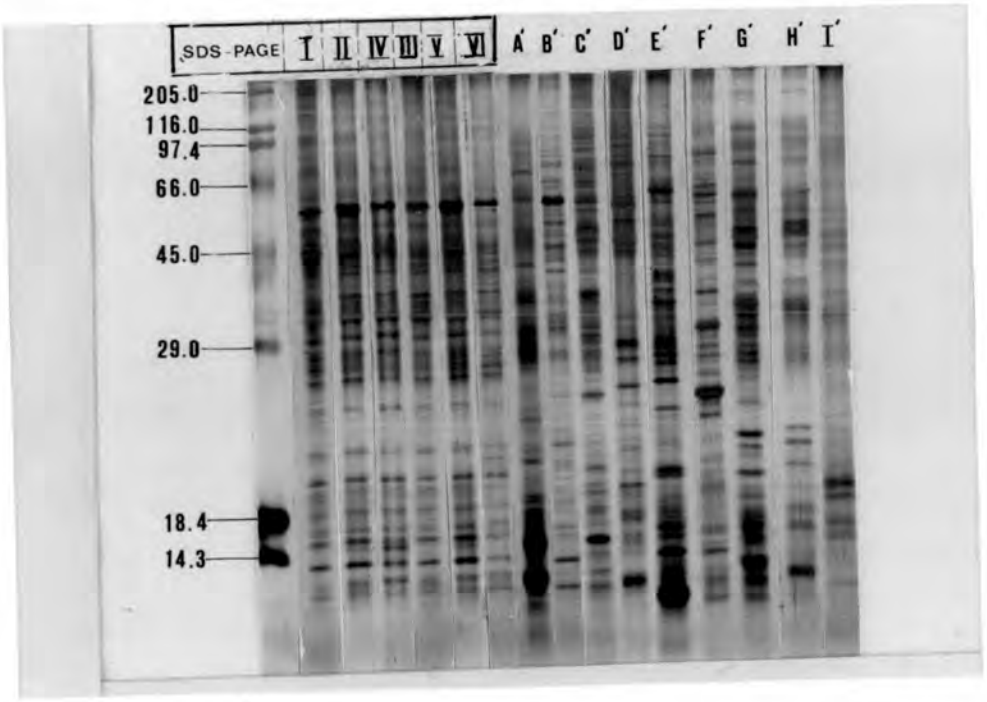


Fig 26 Silver stained SDS-PAGE profile of sonic extract of *P. pseudomallei* Type I-VI and other bacteria;

A' = *P. aeruginosa* ATCC 27853

B' = *P. cepacia* JCM 5510

C' = *P. stutzeri* JCM 5965

D' = *P. putida* JCM 6160

E' = *P. maltophila* JCM 3801

F' = *V. cholerae* 569B

G' = *S. typhi* NCTC 781

H' = *E. coli* ACTCC 25923

I' = *S. aureus* ATCC 25923

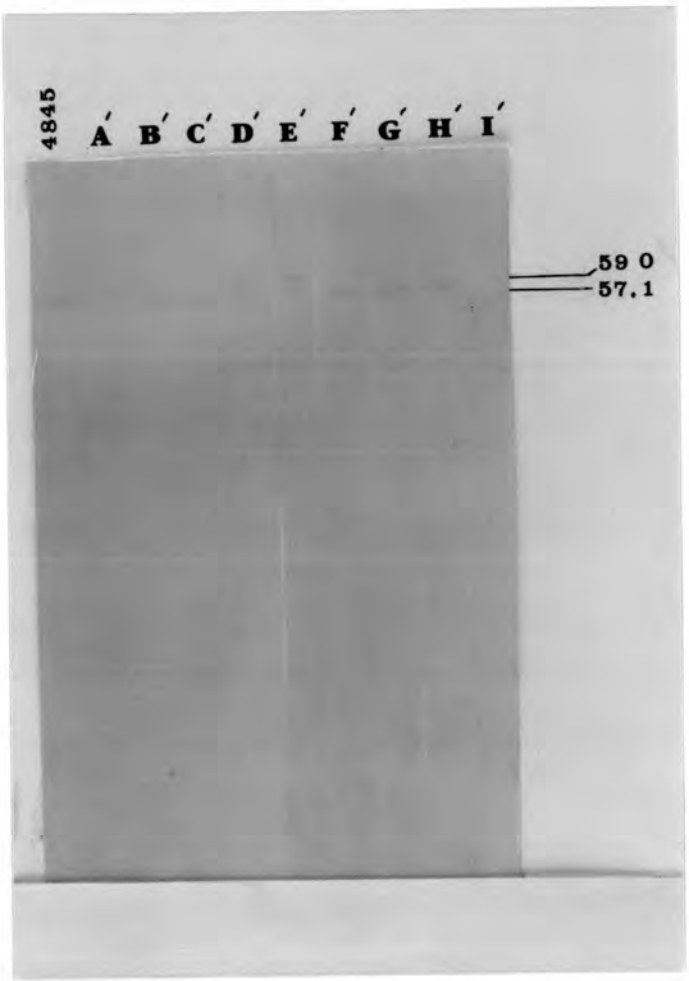


Fig 27 Immunoblot reaction with normal rabbit Ig: of *P. pseudomallei* NCTC 4845 and other bacteria;

A′ = *P. aeruginosa* ATCC 27853

B′ = *P. cepacia* JCM 5510

C′ = *P. stutzeri* JCM 5965

D′ = *P. putida* JCM 6160

E′= *P. maltophila* JCM 3801

F′= *V. cholerae* 569B

G′= *S. typhi* NCTC 781

H′= *E. coli* ATCC 25922

I′= *S. aureus* ATCC 25923

The antigenic patterns of these bacteria the reacted with rabbit antiserum to *P. pseudomallei* NCTC 4845 as shown in Fig 28. The bands appeared in the strip of *P. pseudomallei* were more intensive than those of other bacteria. Some antigenic band of *P. pseudomallei* were in those of bacteria as summarized in Table 13.

Interestingly, the bands with molecular weight of 16.8, 20.7, 24.1, 107.0, 115.0 and 140.0 Kd were antigenic in common in every strain of *P. pseudomallei*. These bands, however, were not seen in the strips of other bacteria.

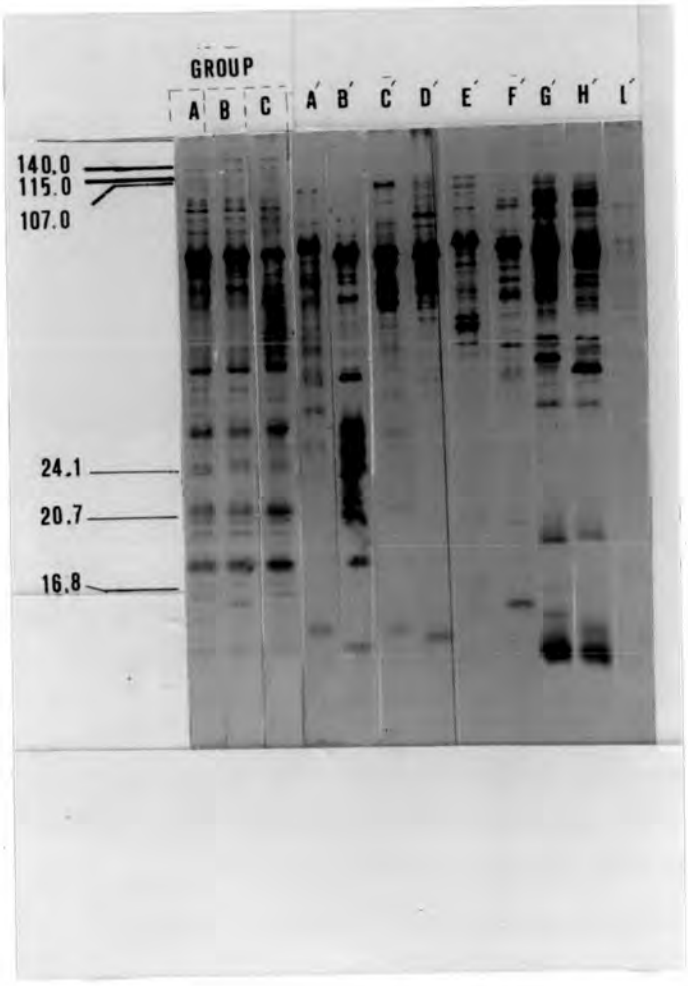


Fig 28 Immunoblot reaction with rabbit antiserum against *P. pseudomallei* NCTC 4845 Ig: of *P. pseudomallei* group A,B,C and other bacteria;

A' = *P. aeruginosa* ATCC 27853

B' = *P. cepacia* JCM 5510

C' = *P. stutzeri* JCM 5965

D' = *P. putida* JCM 6160

E = *P. maltophila* JCM 3801

F'= *V. cholerae* 569B

G'= *S. typhi* NCTC 781

H'= *E. coli* ATCC 25922

I'= *S. aureus* ATCC 25923

Table 13 Summary of the pattern of bands of *P. pseudomallei* which cross reacted with other bacteria in immunoblot

Presence of band MW (Kd)	*P. pseudomallei* * group A	*P. pseudomallei* * group B	*P. pseudomallei* * group C	*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	*S. aureus* ATCC 25923
102.0	+	+	+					+				
90.0	+	+	+					+				
79.0	+	+	+						+		+	
73.0	+	+	+						+	+	+	
67.0	+	+	+				+		+			
64.0	+	+	+	+	+		+	+				
57.1	+	+	+		+	+	+		+	+	+	+
56.0	+	+	-	+		+		+	+	+	+	
52.5	+	+	+	+	+	+	+	+	+	+	+	
49.1	+	+	+		+	+	+	+	+	+	+	
48.8	+	+	+	+		+	+	+		+		
46.5	+	+	+	+	+	+	+					+
44.6	+	+	+			+		+	+	+	+	+
43.0	+	+	+	+	+			+	+	+	+	
37.0	+	+	+	+	+							
34.2	+	+	+		+					+		
31.0	+	+	+						+			
28.5	+	+	+									
27.5	+	+	+		+	+						
27.2	+	+	+		+							
26.4	+	+	+		+							
25.0	+	+	+		+							
22.4	+	+	+		+							
20.4	+	+	+		+							
19.6	+	+	+									
18.6	+	+	+		+							
14.4	-	+	-					+				
13.9	+	+	+									
12.6	+	+	+				+					
12.0	+	+	+		+							

+ = present of band

* each group of *P. pseudomallei* by immunoblot technique