ANTIGENIC DIFFERENCES BETWEEN STRAINS OF *PSEUDOMONAS PSEUDOMALLEI*

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ABSTRACT

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A number of antigens are shared among all of the *Pseudomonas pseudomallei* strains tested due to the occurrence of cross- reactions between the strains. The cross-reaction indices show that the human and soil strains are closely related to each other, as well as the goat and pig strains. However, the human and soil strains are quite different from the sheep strain, while the goat and pig strains are intermediately related to the human and sheep strains. From the results of the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of *P. pseudomallei* antigens tested, it can be concluded that *P. pseudomallei* is characterised by three protein antigens at molecular weights (MWs) of 25 K, 37 K and 38 K. Furthermore, the human strain was specified by proteins at MWs of 32 K, 42 K, 52 K and 64 K, while the non-human strains by proteins at MWs of 39 K and 59 K. The relatively avirulent sheep strain did not possess a protein at MW of 27 K which may be of a virulence protein, probably a superoxide dismutase of *P. pseudomallei*. The immunoblotting of these SDS-PAGE separated protein antigens with each of the immune sera has shown antigenic differences between strains of *P. pseudomallei* quantitatively, and another component of the outer membrane of *P. pseudomallei* (possibly lipopolysaccharide) may contribute to the antigenic differences qualitatively.

Key words: *P. pseudomallei* strains, antigenic differences.

ABSTRAK

Indriana, J. dan R.G. Hirst. 1992. Perbedaan antigenik beberapa strain *Pseudomonas pseudomallei*. *Penyakit Hewan* 24(44): 93-102.

Semua galur *Pseudomonas pseudomallei* yang diuji mempunyai persamaan sejumlah antigen karena adanya reaksi silang diantara galur-galur tersebut. Index reaksi silang menunjukkan bahwa galur manusia dan galur tanah berhubungan erat satu sama lain, demikian juga galur kambing dan galur babi. Tetapi galur manusia dan galur tanah sangat berbeda dengan galur domba, sedangkan hubungan galur kambing dan galur babi dengan galur manusia dan galur domba adalah secara intermediate. Dari hasil pemeriksaan sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) dari antigen-antigen *P. pseudomallei* yang diuji, dapat disimpulkan bahwa *P. pseudomallei* dikarakterisasikan oleh tiga antigen protein pada berat molekul 25 K, 37 K dan 38 K. Lebih lanjut, galur manusia tersifat oleh protein-protein pada berat molekul 32 K, 42 K, 52 K dan 64 K, sedangkan galur-galur non-manusia oleh protein-protein pada berat molekul 39 K dan 59 K. Galur domba yang secara relatif tidak virulen, tidak mempunyai protein pada berat molekul 27 K yang mungkin adalah suatu protein yang virulen, yaitu superoxide dismutase daripada *P. pseudomallei*. Immunoblotting daripada antigen-antigen protein yang dipisahkan secara SDS-PAGE ini dengan setiap serum kebalnya menunjukkan perbedaan-perbedaan antigenik diantara galur-galur *P. pseudomallei* secara kuantitatif, sedang komponen lain dari selaput luar kuman *P. pseudomallei* (kimungkinan lipopolisakarida) menunjukkan perbedaan-perbedaan antigenik tersebut secara kualitatif.

Kata-kata kunci: galur *P. pseudomallei*, perbedaan antigenik.

INTRODUCTION

Pseudomonas pseudomallei, a Gram-negative bacillus, motile, non-acidfast, non-sporebearing, which shows a bipolar on Gram strain reaction, is the causative organism of a glanders-like disease called melioidosis.

It is known that melioidosis will infect humans, domestic and wild animals. All cases of melioidosis have been reported mainly from tropical and subtropical areas between latitudes 20°C north and 20°C south of the equator (Redfearn *et al.*, 1966; Howe *et al.*, 1971) with Southeast Asian Countries (including Indonesia), Northern Australia, Guam and Madagascar as the endemic foci (Howe *et al.*, 1971; Groves, 1979).

Assays of the humoral immune response to melioidosis in the animal host have been performed by the serum agglutination (SA) (Lewis and Olds, 1952; Olds and Lewis, 1954), the fluorescent antibody (FA) (Ashdown, 1981), the complement fixation (CF) (Nigg and Johnston, 1961; Cook, 1962; Nigg, 1963; Laws, 1967a, 1967b; Thomas *et al.*, 1988), and the indirect (passive) haemagglutination (IHA) tests (Ileri, 1965; Laws, 1967a, 1967b; Thomas *et al.*, 1988).

Indirect haemagglutination (IHA) is the test recommended by the Center of Disease Control, Atlanta, Georgia, USA for the serological diagnosis of melioidosis (Ashdown, 1987), and generally is the only serological test available in endemic areas due to its high sensitivity and specificity, cheapness and ease of application. However, negative reactions sometimes occur in culture positive patients (Guard *et al.*, 1984), and IHA titres are of no value in differentiating active from latent infection (Alexander *et al.*, 1970; Ashdown, 1987). Consequently, its use for the serodiagnosis of melioidosis has its limitations (Khupulsup and Petchclai, 1986).

Enzyme linked immunosorbent assay (ELISA), an advanced method for the detection of both antibody and antigen pioneered by Engvall and Perlmann (1971) and has been applied to the diagnosis of many diseases (Keren, 1979; Thiermann and Garrett, 1983; Lee *et al.*, 1985; Schaber *et al.*, 1989). Enzyme linked immunosorbent assay has been studied for the serological diagnosis of human melioidosis (Ashdown *et al.*, 1980; Ashdown, 1982) and it showed excellent sensitivity and specificity, being at least 10 times more responsive than the CF test. However, ELISA studies have not been reported for the animal host.

Variation in the virulence of *P. pseudomallei* strains has been previously described (Indriana and Hirst, in press). It is possible that there may be differences in the proteins expressed by these different *P. pseudomallei* strains. Therefore, an analysis of the proteins of P. pseudomallei was performed using SDS-PAGE. However, this technique per se gives no information on the number of immunoreacting antigens. To date, there are no reports on different protein antigens involved in immunity to *P. pseudomallei.* Such information would be valuable for the immunodiagnosis of and immunisation against melioidosis.

This paper describes the development and the application of an ELISA for studying the cross-reactions among *P. pseudomallei* strains. The proteins were separated by SDS-PAGE, and the immunoreactive *P. pseudomallei* protein antigens were studied on nitrocellulose immunoblots using immune rabbit sera.

MATERIALS AND METHODS

Strains of *Pseudomonas pseudomallei*

The human (AN) isolate of *P. pseudomallei* used was obtained from Townsville General Hospital, Australia. The soil (C2) isolate and the sheep (J53), goat (X1003) and pig (1328) isolates were obtained from Oonoonba Veterinary Laboratory, Townsville, Australia.

Bacterial antigens

Heat-killed whole cell antigens

For immunising rabbits for the purpose of producing hyperimmune sera, the antigens were prepared by the methods of Laws (1967a) and Ashdown (1982) with some modification. One fresh single colony (about 1 mm in diameter) of each *P. pseudomallei* strain was inoculated into 3 ml Brain Heart Infusion (BHI) broth and incubated for 4 hours at 37°C. This bacterial suspension was used to inoculate BHI agar plates by pouring the organisms over the agar surface evenly to obtain confluent growth. The excess suspension was withdrawn with a sterile Pasteur pipette, then the plates were incubated overnight at 37°C. The confluent growth of each strain was harvested in sterile distilled water (3 ml for each plate), pooled, then centrifuged at 10,000 g (Beckman Model J2-21 centrifuge, rotor JA-17) for 10 minutes at 4°C. The supernate was removed, the pellet was resuspended in sterile distilled water and the cells were standardised to 144 mg/ml wet cell weight, then stored at 4°C. Its purity was checked by subculturing on to blood agar and MacConkey agar. Provided the subculture results were satisfactory, the organisms were killed by heating in a waterbath at 85°C for one hour. The sterility of the preparation was determined by subculturing on to a BHI agar plate and incubated for 4 days at 37°C.

Heat-killed whole cell sonicate antigens

The heat-killed cell suspension prepared was disrupted by ultrasonication performed continuously on ice for 10 minutes (MSE Ultrasonic Disintegrator, Thomas Optical and Scientific Co Pty Ltd, Melbourne, Australia; output control 6-7). Cellular debris was removed by centrifugation at 10,000 g for 30 minutes at 4°C. The supernate (antigen) was filtered through 0.8 um and 0.2 um pore-size sterile filters, then distributed aseptically in sterile 5 ml containers and stored at -20°C for use in ELISA.

Hyperimmune sera

Hyperimmune sera against heat-killed whole cells of *P. pseudomallei* strains were raised in LOP hybrid male rabbits. These rabbits were obtained from Central Animal Breeding House, University of Queensland Veterinary Farm, Brisbane, Australia. The heat-killed

whole cells described above were adjusted to a protein concentration of 2 mg/ml using then 0.5 ml of this suspension was emulsified with 0.5 ml of Freund's complete adjuvant, followed by sonication on ice for 10 seconds to help form the emulsion. This preparation was re-emulsified with an equal volume of sterile physiological saline containing 2% Tween-80, and injected intramuscularly into the left and right hind legs of each rabbit. This double type emulsion (water-in oil-in water) is more effective than the water-in oil type emulsion because it is more stable and less viscous, thus easier to administer and produces less local reactions (Smith, 1988).

Prior to immunisation, all of the rabbits were bled and their sera were used as the non-immune control sera.

After approximately 30 days, a booster injection was given subcutaneously using in this instance Freund's incomplete adjuvant. Rabbits were bled from the medial ear artery, 10-20 ml at 10, 14 and 18 days after the booster injection. The sera were separated from the clot and centrifuged at 1,500 rpm for 10 minutes to remove the residual red blood cells, then distributed into 5 ml sterile screw-capped plastic tubes and stored at -20°C.

Serological cross-reactions among Pseudomonas pseudomallei strains demonstrated by enzyme linked immunosorbent assay

This assay was carried out using disposable 96 wells U-bottom polyvinylchloride (PVC) microtitre plates (ICN Biomedicals, Sydney, Australia) as the support for the solid-phase antigens.

Goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad Laboratories) was used in this assay.

The substrate was 0.1 M citrate phosphate buffer pH 4.2, 1 mM 2.2 - Azino-di-[3-ethyl-benzthiazolinsulfonat (6)] (ABTS - Boehringer Mannheim, Sydney, Australia), and 2.5 mM hydrogen peroxide.

Optimisation of enzyme linked immunosorbent assay conditions

A chequerboard titration was performed to determine the optimal dilution of each antigen and the conjugate.

Briefly, serial two-fold dilutions of each antigen were made in carbonate coating buffer to correspond to its protein concentration which ranged from 520 ng/ml to 0.3 ng/ml, 480 ng/ml to 0.2 ng/ml to 0.2 ng/ml, 540 ng/ml to 0.3 ng/ml, 760 ng/ml to 0.4 ng/ml, and 640 ng/ml to 0.3 ng/ml of human, soil, sheep, goat and pig strains respectively. One hundred microlitres of each antigen dilution was added to the wells of the microtitre plates, and incubated overnight (approximately 16 hours) at 4°C. These antigen-coated microtitre plates were used on that day, and subsequently were washed 3 times with phosphate buffered saline with Tween-20 (PBST), tapped and drained between each wash.

Both pre- and post-immunisation sera were diluted 1:100 in T.E.N. buffer with Tween-20 and Casein (Ten-T-C), then 50 *ul* volumes of the diluted sera were added in duplicate to the wells of the microtitre plates, followed by incubation at room temperature for one hour. The plates were then washed 3 times as above.

The conjugate was diluted in Ten-T-C, and a serial dilution of 1:2000, 1:3000, 1:4000 and 1:5000 was prepared, then 50 *ul* of each dilution was added to the plates, incubated at room temperature for one hour, then washed 3 times as described previously.

Substrate was added in volumes of 100 *ul* to each well and the plates were incubated at room temperature for one hour.

Another microtitre plate was used for the control (blank), and substrate was added to the first column of the plate. No serum was added.

The optical density (OD) values were read at 414 nm in an ELISA plate reader (Titertek Multiskan MCC, Labsystems, ICN Biomedicals).

Antiserum assays

Each well of a microtitre plate was coated with 100 ul of the test antigen diluted to its optimal concentration in carbonate buffer, and incubatedd overnight at 4°C. The antigen coated microtitre plates were used on that day. The plates were then washed 3 times with PBST, tapped and drained between each wash.

Fifty microlitres of each test serum was diluted by serial two- fold dilutions in Ten-T-C ranging from 1:25 to 1:51,200 into duplicate wells of the homologous antigen and incubated at room temperature for one hour. Using PBST, the wells were washed again 3 times as decribed above.

Substrate in aliquots of 100 *ul* was added to each well. After one hour incubation at room temperature, the enzyme-substrate reaction was read in an ELISA plate reader at 414 nm.

A similar amount of substrate was added to the first column of another microtitre plate. No serum was added. This constituted the control (blank).

Cross-reaction assays

To compare the degree of cross-reactivity between strains, an indirect ELISA was adapted using heat-killed

whole cell sonicates of each of the *P. pseudomallei* strains for use with each of the antisera.

Each *P. pseudomallei* antigen was diluted to its optimal concentration in coating buffer. Using two microtitre plates, each antigen in 100 *u*l aliquots was coated to the wells as follows:

- Human (AN) strain antigen was added to wells in columns 1 and 2
- Soil (C2) strain antigen to wells in columns 3 and 4
- Sheep (J53) strain antigen to wells in columns 5 and 6
- Goat (X1003) strain antigen to wells in columns 7 and 8
- Pig (1328) strain antigen to wells in columns 9 and 10
- Wells in columns 11 and 12 were controls, to which only substrate was added.

The microtitre plates were incubated overnight at 4°C and were used on the following day. Following three washings of the microtitre plates with PBST, each test serum was diluted to its optimal concentration as determined above, of 1:400 in Ten-T-C and dispensed in 50 *u*l volumes in duplicate across the microtitre plates in such a way that rows A and B were used for testing the human strain antisera, row A for the reactive serum and row B for the non-reactive control serum. Similarly, rows C and D for the soil strain reactive and non-reactive antisera, rows E and F for the sheep strain reactive and non-reactive antisera, rows G and H for the goat strain reactive and non-reactive antisera, and rows A and B of another microtitre plate for the pig strain reactive and non-reactive antisera respectively. Then the plates were incubated for one hour at room temperature and subsequent washings with PBST were again repeated as previously described.

Conjugate at the optimal dilution (1:3000 in Ten-T-C) in 50 *u*l aliquots was added to the wells, followed by one hour incubation at room temperature.

The microtitre plates were again washed 3 times with PBST as described above, then substrate in 100 ul aliquots was added. The OD values were read after one hour incubation at room temperature in an ELISA plate reader at 414 nm.

Recognition of *Pseudomonas pseudomallei* antigens by rabbit antisera on nitrocellulose immunoblots

Antigens for SDS-PAGE

Whole cell lysate antigens of each of the *P. pseudomallei* strains were prepared by sonicating the heat-killed whole cell antigen in 1% sodium dodecyl sulphate.

SDS-PAGE

The electrophoresis procedure used was performed as described by Laemmli (1970). The protein concentrations of the antigens were adjusted to 4 mg/ml, and then were diluted to 3:1 with sample buffer, boiled for 5 minutes, and 10 ul was added to the well of the gel, that is, approximately 30 ug of protein antigen. Pre-stained low molecular weight (MW) standards (Bio-Rad Laboratories) were used in the immunoblot studies. These consisted of phosphorylase b (110,000 MW), bovine serum albumin (84,000 MW), ovalbumin (47,000 MW), carbonic anhydrase (33,000 MW), soybean trypsin inhibitor (24,000 MW), and lysozyme (16,000 MW). The MW standards were plotted onto a graph with the distance (mm) of each standard band from the top margin of the separating gel on the X axis and the MWs of each on the Y axis. By measuring the distance of each protein band for each antigen, the MW of the protein antigens was determined.

Electrophoretic transfer

After electrophoresis of the five whole cell lysate antigens, the gels were equilibrated in transfer buffer for 30 minutes. Electrophoretic transfer was performed in a transblot cell (Bio- Rad Laboratories). The gels were laid on top of sponge pads and filter papers, which had previously been wetted with transfer buffer. Pre-wetted 0.45 um pore-size nitrocellulose membranes (Bio-Rad Laboratories) were then placed on top of the gels in such a way that no air bubbles appeared between the gel and the membrane. Subsequently, the gel and nitrocellulose stack was overlaid with pre-wetted filter papers and sponge pads, clamped together and placed in the transblot cell containing transfer buffer, with nitrocellulose membranes toward the anode. The transfer was run at a constant voltage of 50 V (Bio-Rad Power Supply Model 250/2.5) for one hour. The nitrocellulose transblots were soaked overnight in Ten-T-C, then washed 3 times in PBST for 15 minutes.

Immunochemical detection of antigens

The nitrocellulose transblots were placed in petri dishes (diameter 85 mm, depth 20 mm) with 10 ml of diluted antisera (1:100 in Ten-T-C) and rocked for one

hour. The transblots were then washed for 15 minutes with three changes of PBST, followed by rocking for one hour with goat-anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad Laboratories) diluted 1:3000 in Ten-T-C. The nitrocellulose transblots were again washed in PBST as above, then reacted with a substrate solution containing hydrogen peroxide and diaminobenzidine by rocking for one minute. The reaction was stopped by adding distilled water.

RESULTS

Serological cross-reactions among Pseudomonas pseudomallei strains demonstrated by enzyme linked immunosorbent assay

Optimal conditions for enzyme linked immunosorbent assay

The ratios between OD values of reactive and non-reactive sera (P/N ratio) for the optimal concentrations of conjugate and antigens are shown in Table 1.

The optimal dilution of conjugate for all of the *P. pseudomallei* antigens was 1:3000. The optimal antigen dilution was that dilution of antigen which gave the highest reactive OD values at 414 nm (at least > 1.4) compared with low non-reactive values (at least < 0.1). On this basis, the optimal dilution of each antigen and its protein concentration was used as follows:

- Human (AN) antigen at dilution 1:20,000 - 130.0 ng/ml
- Soil (C2) antigen at dilution 1:100,000 - 24.0 ng/ml
- Sheep (J53) antigen at dilution 1:200,000 - 13.5 ng/ml
- Goat (X1003) antigen at dilution 1:250,000 - 15.2 ng/ml
- Pig (1328) antigen at dilution 1:250,000 - 12.8 ng/ml

Table 1. The P/N ratios for the optimal antigen dilutions obtained from *Pseudomonas pseudomallei* strains

	Antigen concentrations (ng/mL)								
	Human			Soil		Sheep		Goat	Pig
	130	30	15	16.9	8.4	23.8	11.9	20	10
P/N ratio at conjugate dilution 1:3000	46.5	97.5	94.5	24	28.6	18.1	22.7	60	57

Antiserum assays

At a dilution of 1:400 when reacted with each of the homologous *P. pseudomallei* antigens, the high titre reactive sera had OD values > 1.2 and the low titre non-reactive sera had OD values < 0.1. The data and the P/N ratios are presented in Table 2. Thus 1:400 was chosen as the optimal serum dilution.

Table 2. The P/N ratios for the optimal serum dilution obtained from *Pseudomonas pseudomallei* strains

Serum dilution	Human (AN)	Soil (C2)	Sheep (J53)	Goat (X1003)	Pig (1328)
1:400	17.0	39.8	123.0	31.8	26.2

Cross-reaction assays

The results presented in Table 3 clearly show that each antiserum reacted strongly, both with its homologous antigen and also with the human and soil antigens.

Table 3. The degree of cross-reactivity of immune rabbit sera to homologous and heterologous *Pseudomonas pseudomallei* antigens

Immune rabbit serum	*P. pseudomallei* antigens				
	Human (AN)	Soil (C2)	Sheep (J53)	Goat (X1003)	Pig (1328)
Human (AN)	+++	+++	++	+++	+++
Soil (C2)	+++	+++	++	+++	+++
Sheep (J53)	+++	+++	+++	++	+
Goat (X1003)	+++	+++	+	+++	++
Pig (1328)	+++	+++	++	+++	+++

Cross-reaction is considered to be:
Strong (+++) OD ≥ 1.0
Moderate (++) OD 0.4 - 1.0
Weak (+) OD < 0.4

The human, soil and pig antisera reacted moderately with the sheep antigen, but reacted strongly with the remaining antigens.

The sheep antiserum reacted moderately with the goat antigen and a weak cross-reaction was observed between the sheep antiserum and the pig antigen.

The goat antiserum reacted weakly with the sheep antigen, and moderately with the pig antigen.

When the proportions of heterologous to homologous OD were assessed (Table 4), the cross-reaction indices (r values) showed values of ≥ 1.0 between the human strain antigen and the sheep, goat and pig strain antiserum, as well as between the soil strain antigen and the human, goat and pig strain antiserum. The pattern of the results presented in Tables 3 and 4 are similar.

Table 4. Cross-reaction indices obtained for each immune rabbit serum to each of the *Pseudomonas pseudomallei* antigens

Immune rabbit serum	*P. pseudomallei* antigens				
	Human (AN)	Soil (C2)	Sheep (J53)	Goat (X1003)	Pig (1328)
Human (AN)	1.0	1.04	0.22	0.56	0.78
Soil (C2)	0.75	1.0	0.41	0.76	0.85
Sheep (J53)	1.34	0.92	1.0	0.54	0.27
Goat (X1003)	1.62	1.77	0.38	1.0	0.86
Pig (1328)	1.29	1.45	0.59	0.94	1.0

The reaction is considered to be homologous when the proportion of heterologous OD to homologous OD (r value) is ≥ 1.0.

$$r\ value = \frac{OD\ heterologous\ reaction}{OD\ homologous\ reation}$$

Recognition of Pseudomonas pseudomallei antigens by rabbit antisera on nitrocellulose immunoblots

SDS-PAGE of Pseudomonas pseudomallei antigens

The electrophoretic analysis of the whole cell lysate protein antigens of *P. pseudomallei* are shown in Figure 1. From the human strain, approximately 14 protein bands were evident, while from the soil, sheep, goat and pig strains, 10, 7, 10 and 10 protein bands were observed respectively. The MWs of those protein bands are presented in Table 5.

Table 5. The molecular weights ($x10^3$) of the protein bands yielded from the whole cell lysate antigens of *Pseudomonas pseudomallei*

P. pseudomallei strains				
Human	Soil	Sheep	Goat	Pig
64	-	-	-	-
-	59	59	59	59
52	-	-	-	-
42	-	-	-	-
-	39	39	39	39
38	38	38	38	38
37	37	37	37	37
33	-	-	-	33
32	-	-	-	-
30	30	-	-	30
27	27	-	27	27
-	-	26	-	-
25	25	25	25	25
22	22	-	22	-
21	21	-	21	-
-	-	20	20	20
19	19	-	19	-
18	-	-	-	18

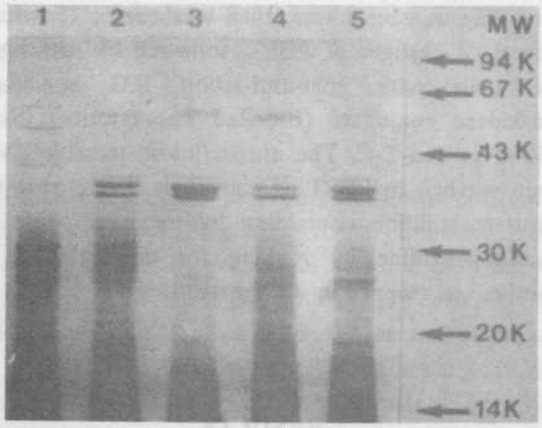

Figure 1. SDS-PAGE of whole cell lysate antigens of *Pseudomonas pseudomallei*

1. Human strain antigen
2. Soil strain antigen
3. Sheep strain antigen
4. Goat strain antigen
5. Pig strain antigen

Immunochemical detection of Pseudomonas pseudomallei antigens on nitrocellulose immunoblots

Figure 2 shows the protein profiles of the *P. pseudomallei* antigens when reacted with the human strain antiserum. Three strong protein bands of homologous strain antigen (lane 1) have been identified at MWs of approximately 38 K, 52 K and 64 K. This human strain antiserum also shows a distinct reaction with protein bands of the soil, sheep, goat and pig strain antigens at MWs of approximately 25 K, 38 K, 39 K and 59 K, but the protein band at MW of 59 K of the sheep strain antigen (lane 3) showed only a slight reaction.

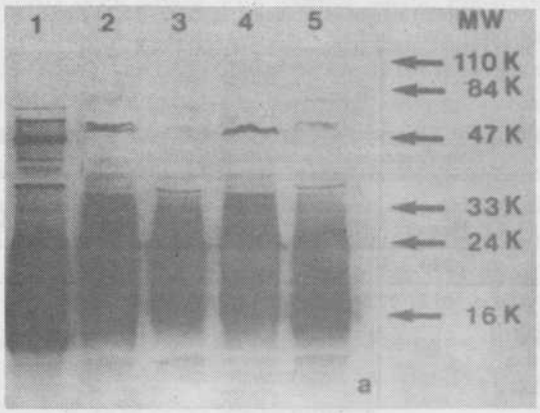

Figure 2. Immunoblot of the protein profiles of *Pseudomonas pseudomallei* antigens using immune rabbit serum against *Pseudomonas pseudomallei* human strain.

1. Human strain antigen
2. Soil strain antigen
3. Sheep strain antigen
4. Goat strain antigen
5. Pig strain antigen

Figure 3 shows the protein profiles of the *P. pseudomallei* antigens when reacted with the soil strain antiserum. Three distinct protein bands of homologous strain antigen (lane 2) have been recognised at MWs of approximately 38 K, 39 K and 59 K. This soil strain antiserum recognised distinctly the protein bands of the human strain antigen (lane 1) at MWs of 25 K, 38 K, 42 K, 52 K and 64 K. The goat strain (lane 4) and the pig strain (lane 5) antigen reactions occurred with protein bands at MWs of 25 K, 38 K, 39 K and 59 K. Similar reactions were observed with the sheep strain antigen, but the reactions with the proteins at MWs of 39 K and 59 K were weak.

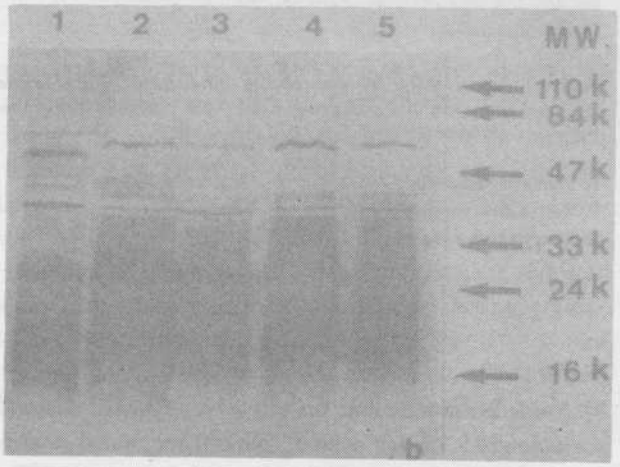

Figure 3. Immunoblot of the protein profiles of *Pseudomonas pseudomallei* antigens using immune rabbit serum against *Pseudomonas pseudomallei* soil strain.

1. Human strain antigen
2. Soil strain antigen
3. Sheep strain antigen
4. Goat strain antigen
5. Pig strain antigen

Figure 4 shows the protein profiles of the *P. pseudomallei* antigens when reacted with the sheep strain antiserum. Two strong protein bands of the homologous strain antigen (lane 3) were identified at MWs of 25 K and 59 K. The sheep strain antiserum only weakly identified the protein band at MW of 39 K. This antiserum recognised strongly four protein bands of the human strain antigen at MWs of 25 K, 38 K, 42 K and 52 K and two bands of the soil strain (lane 2) and goat strain (lane 4) antigens at MWs of 25 K and 59 K. Two protein bands at MWs of 38 K and 59 K of the pig strain antigen (lane 5) were faintly identified, however, the protein band at MW of 25 K was distinctly demonstrated.

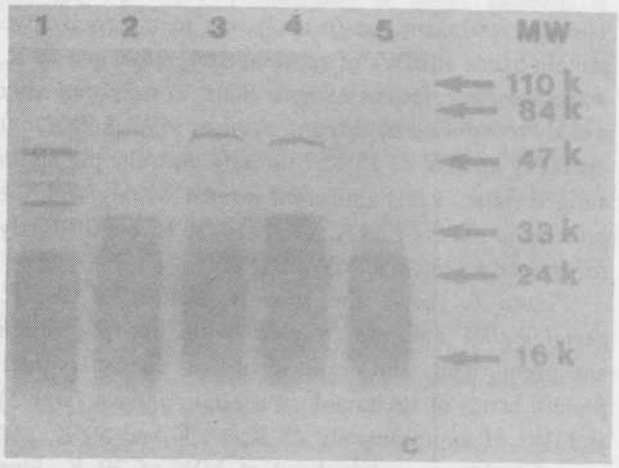

Figure 4. Immunoblot of the protein profiles of *Pseudomonas pseudomallei* antigens using immune rabbit serum against *Pseudomonas pseudomallei* sheep strain

1. Human strain antigen
2. Soil strain antigen
3. Sheep strain antigen
4. Goat strain antigen
5. Pig strain antigen

Figure 5 shows the protein profiles of the *P. pseudomallei* antigens when reacted with the goat strain antiserum. One strong protein band of homologous strain antigen (lane 4) was recognised at a MW of 59 K and very weakly identified a protein band at MW of 39 K. However, a protein band at MW of 25 K was distinctly recognised. This goat strain antiserum identified protein bands of the human strain antigen (lane 1) at MWs of 25 K, 38 K, 42 K, 52 K and 64 K.

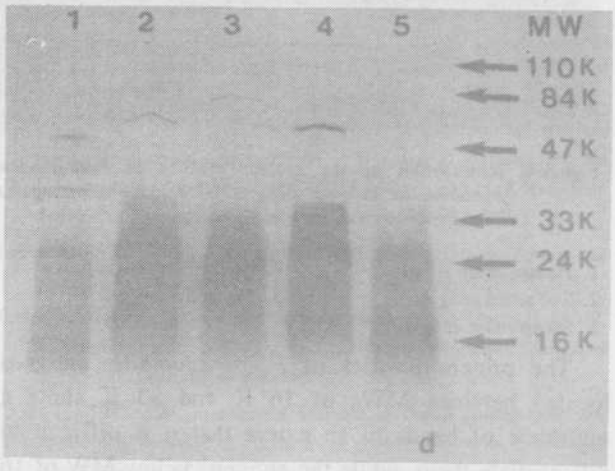

Figure 5. Immunoblot of the protein profiles of *Pseudomonas pseudomallei* antigens using rabbit immune serum against *Pseudomonas pseudomallei* goat strain

1. Human strain antigen
2. Soil strain antigen
3. Sheep strain antigen
4. Goat strain antigen
5. Pig strain antigen

The soil strain antigen (lane 2) was identified by two protein bands at MWs of approximately 25 K and 59 K, while the sheep strain antigen (lane 3) has been very weakly recognised by this antiserum at MW of 59 K, but distinctly at MW of 25 K. Similarly with the pig strain antigen (lane 5) this antiserum reacted weakly with the proteins at MWs of 38 K, 39 K and 59 K, but distinctly recognised a protein at MW of 25 K.

Figure 6 shows the protein profiles of the *P. pseudomallei* antigens reacted with the pig strain antiserum. This antiserum identified distinctly three protein bands of its homologous strain antigen (lane 5) at MWs of approximately 25 K, 38 K and 59 K, and verey weakly a protein band at MW of 39 K. Five distinct protein bands at MWs of 25 K, 38 K, 42 K, 52 K and 64 K of human strain antigen (lane 1) were identified by this pig strain antiserum, and four protein bands of the soil strain (lane 2) and the goat strain (lane 4) antigens were recognised at MWs of 25 K, 38 K, 39 K and 59 K. Two distinct bands at MWs of 25 K and 38 K were identified from the sheep strain antigen (lane 3).

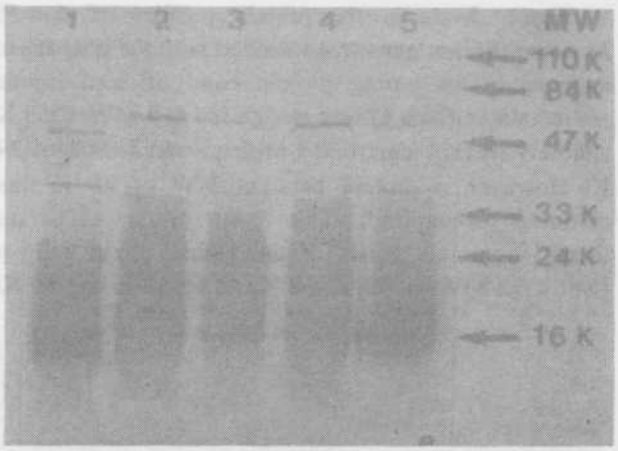

Figure 6. Immunoblot of the protein profiles of *Pseudomonas pseudomallei* antigens using rabbit immune serum against *Pseudomonas pseudomallei* pig strain

1. Human strain antigen
2. Soil strain antigen
3. Sheep strain antigen
4. Goat strain antigen
5. Pig strain antigen

The protein profiles of *P. pseudomallei* antigens located between MWs of 16 K and 33 K show a multitude of bands to an extent that it is difficult to differentiate them, while the antigens below MW of 16 K are probably lipopolysaccharide. However, it seems that this group of proteins (between MWs of 16 K and 33 K) is common to all of *P. pseudomallei* antigens and only a protein band at MW of approximately 25 K was distinctly identified.

Figure 7 shows the immunoblots of these separated antigens which were reacted with pre-immunisation serum. No reaction was observed.

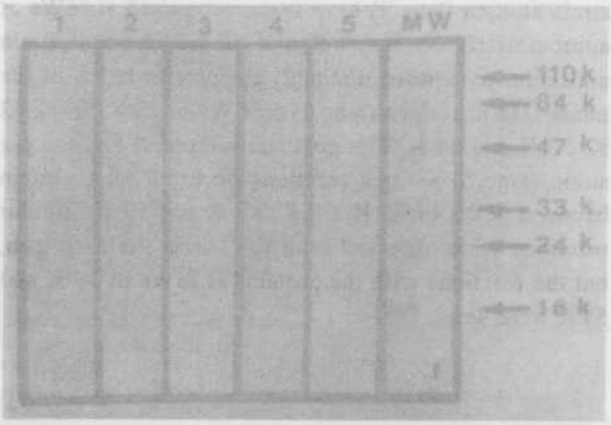

Figure 7. Immunoblot of the protein profiles of *Pseudomonas pseudomallei* antigens using pre-immunisation rabbit immune

1. Human strain antigen
2. Soil strain antigen
3. Sheep strain antigen
4. Goat strain antigen
5. Pig strain antigen

DISCUSSION

Some cross-reactions occurred between all of the *P. pseudomallei* strains tested, which suggested that a number of antigens are shared among these strains. The cross-reaction indices, however, suggest the human and soil strains are closely related, and quite different from the sheep strain. The goat and pig strains while being closely related to each other, are intermediate in their relationship with the human and sheep strains.

An analysis of the protein antigens by SDS-PAGE indicated that the protein antigens common to all strains appear to be at MWs of 25 K, 37 K and 38 K. This suggests that *P. pseudomallei* is characterised by at least these three proteins. The proteins characteristic of P. pseudomallei strains of non-human origin, other than these three bands, appear to be at MWs of 39 K and 59 K. The human strain was specified by proteins at MWs of 32 K, 42 K, 52 K and 64 K. Interstingly, the sheep strain did not possess a protein at MW of 27 K, but yielded one at MW of 25 K instead. The protein at MW of 27 K may be a virulence protein, possibly a superoxide dismutase (SOD) - protein of *P. pseudomallei*, as the subunit SOD-protein of *Nocardia asteroides* was reported to have a MW of 25 K (Beaman and Beaman, 1990).

However, when these SDS-PAGE separated protein antigen were blotted with each of the immune sera, many antigens were shared with differences in the intensity of staining. This probably reflects quantitative differences only. Only in the human strain were antigens at MWs of 42 K, 52 K and 64 K demonstrated by SDS-PAGE. However, these human strain antigens reacted when immunoblotted with antisera from each of the other strain antisera. Furthermore, the human strain antiserum only detected these specific MW proteins in the homologous strain immunoblot reaction. This implies that the proteins at MWs of 42 K, 52 K and 64 K were present in the heat-killed whole antigen preparation but had been destroyed when the sheep, goat, pig and soil antigens were sonicated in 1% SDS prior to their electrophoretic separation by SDS-PAGE.

Other components of the outer membrane of *P. pseudomallei* may contribute to the qualitative differences observed. Lipopolysaccharide (LPS) may thus the responsible for the strain differences reported (Stranton and Fletcher, 1932; Miller *et al.*, 1948; de Lajudie and Brygoo, 1953; Laws and Hall, 1963; Laws and Mahoney, 1964). The failure to detect some infections by the CF test would suggest that this test relies predominantly on LPS antigen. The apparently higher sensitivity of the IHA reported (Jones and Hambie, 1976; Thin, 1976; Thomas *et al.*, 1988) would suggest that protein antigens form the basis of the latter test.

This study has thus shown qualitative and quantitative antigenic differences between strains of *P. pseudomallei*. These differences may be in part due to the LPS of the outer membrane of the organism. They may also result from the method of antigen preparation. It has also highlighted the need to use purified components of the cell wall for serotyping, studies, and that protein antigens should give the greatest sensitivity in diagnostic tests. Further work is required to elucidate those points and to investigate the predominant antigent in natural infection by immunoblotting protein and LPS antigens with convalescent sera. This latter study would provide useful information on candidate immunogens for use in a subunit vaccine.

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