DNA fingerprinting of human isolates of *Burkholderia pseudomallei* from different geographical regions of Malaysia

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Abstract. Melioidosis is an infectious disease caused by *Burkholderia pseudomallei* and endemic in Southeast Asia. One hundred and forty six clinical isolates of *B. pseudomallei* from different states in Malaysia were obtained and molecular typing was carried out using pulsed-field gel electrophoresis (PFGE). Overall, nine clusters were successfully identified. *Burkholderia pseudomallei* isolates used in this study were found to be genetically diverse and there were differences in the clusters of isolates from peninsular and east Malaysia. BS9 cluster was the most common cluster and found in all the states while BS2 cluster only existed in a particular state. Based on the PFGE analysis, the distribution of different *B. pseudomallei* clinical isolates in Malaysia was mapped.

INTRODUCTION

Melioidosis is one of the few genuinely tropical infections that is well established in Southeast Asia and Northern Australia. The disease is caused by *Burkholderia pseudomallei* which is a gram negative saprophytic bacillus found in soil and water, but found to persist in some tropical regions better than others, such as hyper endemic foci or "hot spots" that are known to exist in North eastern Thailand and in the states of Sabah and Sarawak in East Malaysia but the specific attributes for this is not known. Some suggestions are the presence of unusually virulent or infective strains, practices such as farming techniques or a particularly high concentration of *B. pseudomallei* in the soil and waters of these regions (Puthucheary & Vadivelu, 2002).

Malaysia is an endemic area for melioidosis but the epidemiology of *B. pseudomallei* in Malaysia is relatively unknown. However, it is known that cases are not evenly distributed, and anecdotally more cases are being reported from the so called "hyper-endemic" states such as Pahang, Kelantan, Sabah and Sarawak. Melioidosis is not a notifiable disease in Malaysia, so there are only a few reports available on the incidence in Malaysia. In Singapore, which is geographically and demographically similar to Malaysia, where melioidosis is a notifiable disease, the mean annual incidence is given as 1.3 per 100,000 population (Heng & Goh, 1994) but in the state of Pahang the annual incidence has been reported as 4.3 per 100,000 population per year; adults 6.0 and paediatric 1.6 per 100,000 respectively (How *et al.*, 2009). Whilst in the southern

state of Johor, only 44 new cases were recorded over a period of 5 years (Pagalavan, 2005) and there were 250 cases reported from the State of Sabah over 7 years.

Nothing is known about bacterial strain variation in determining the severity and pattern of disease although differential virulence and tropism have been indicated but no large studies have been carried out for definite conclusions. Understanding the epidemiology of melioidosis can be undertaken by molecular typing which offers a very discriminatory tool for differentiating such strains which are phenotypically similar. Published reports have shown that DNA macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) enables greater discrimination between phenotypically identical isolates, evidence of genetic relatedness of these strains if any and probable clonality of "identical" strains (Vadivelu *et al.*, 1997).

To date, we have a large collection of strains obtained from sporadic cases of melioidosis from different geographical locations (states) within Malaysia and with genome macrorestriction finger-printing resolved by pulsed-field gel electrophoresis (PFGE) and cluster analysis; this may allow us to clarify whether there are predominant genotypes associated with certain geographical locations.

MATERIALS AND METHODS

Bacterial strains

A total of 146 isolates of *B. pseudomallei* from the Medical Microbiology Department, University of Malaya, Kuala Lumpur, Malaysia, were used in this study.

These strains were isolated from sporadic cases of melioidosis from 1980 to 2008 in different regions in Malaysia. Specimens included blood, pus, tissues and body fluids, urine, sputum and peritoneal dialysates. They had been identified, confirmed by routine laboratory methods and cryopreserved in 20% glycerol at -80ºC. Working cultures were maintained in Luria-Bertani (LB) agar and broth. Safety

precautions were taken in working with these organisms, including the use of a biohazard safety cabinet and gloves.

Analytical Profile Index (API20NE) test

The Analytical Profile Index (API20NE) system (BioMerieux, Lyon, France) was also applied in study for further confirmation of *B. pseudomallei* cultures before PFGE profiling was carried out. The test was performed at 30ºC according to the manufacturer's instructions. The results of each test were recorded as positive or negative. All tests were further scored according to the manufacturer's interpretive colour chart. Numerical profiles were interpreted using the APILAB plus software version 3.3.3.

Preparation of genomic DNA

DNA for pulsed-field gel electrophoresis (PFGE) was prepared according to the CDC One-Day Standard Laboratory Protocol for Molecular subtyping described by Ribot *et al.* (2006). Briefly, *B. pseudomallei* isolates were grown overnight and bacterial cell suspensions were adjusted spectrophotometrically to an optical density of 1.3 – 1.4 at 610 nm. Agarose plugs (1.6% (w/v) Seakem Gold Agarose, Rockland, Maine) were prepared and were lysed at 50ºC overnight in 2 ml of lysis buffer (50 mM Tris, 50 mM EDTA, 1% (w/v) sodium lauroyl-sarcosine and 20 mg/ml of Proteinase K). The plugs were then washed twice with 15 ml deionised water and 4 times with 10 ml of TE buffer. The plugs were then cut into 2 mm slices and digested overnight with restriction enzyme *Spe*I (New England BioLabs, USA). *Salmonella* Braenderup H9812 (CDC, Atlanta, USA) and Lamda DNA concatamers were used as molecular standards in this study.

Electrophoresis

The restricted DNA fragments were separated by electrophoresis using a CHEF DR II (Bio-Rad Laboratories, USA) according to a rapid approach as described by See *et al.* (2009). After that, gels were

stained, destained and visualized under UV light using a Gel Doc 2000 system (Bio-Rad Laboratories, USA). DNA fingerprints were stored and exported to BioNumerics software version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium), where a dendrogram was created using Dice coefficient based on the algorithim unweighted pair group method with arithmetic mean (UPGMA). The genetic diversity and relatedness of the *B. pseudomallei* isolates were compared at 85% similarity at 1% optimization and 1.8% tolerance values.

RESULTS

The 146 isolates of *B. pseudomallei* used in this study came from patients who originated from all States in Malaysia except the 3 states of Negeri Sembilan, Malacca and Perlis. The distribution of these is shown in Table 1. The majority of the strains (61%) came from septicaemic patients.

There were 41 cases that originated from Kuala Lumpur and Selangor. This is the capital city and the most densely populated state in Malaysia as well as the location of the University of Malaya where this study was undertaken. Next were the east coast states of Pahang -18 followed by 17 from Trengganu and 16 from Kedah. No isolates were available from Negeri Sembilan, Malacca or Perlis. The sources of the 146 *B. pseudomallei* isolates are shown in Table 1, and most came from blood and pus specimens (Figure 2).

In keeping with published reports the most frequent age group was from 40 to 60 years but the range was from less than 10 years to 80 years. The gender distribution was 3.6 males to one female. Ethnically Malays formed the largest group (52.7%), followed by Chinese (13.7%) and Indians (12.3%) and the ethnicity was not available for 21.2% of the patients. The ethnic ratio is a reflection of the racial composition of Malaysia.

The API20NE tests correctly identified all the 146 bacterial isolates as *B. pseudomallei* and the profiles obtained are shown in Figure 2. For PFGE profiling, *Spe*I was used to digest genomic DNA of the *B. pseudomallei* isolates, the resulting PFGE profiles (PFPs) were assigned arbitrary designation and analysed by Bionumerics software. The choice of restriction enzyme (RE) depends on the G+C content of the organism which is 64% for *B. pseudomallei* and RE *Spe*I (5'-ACTAGT-'3) was found to be suitable which was able to generate less than 20 fragments (Mohandas *et al.*, 1994).

All the isolates were typeable and identical banding patterns were obtained when repeated analysis were carried out. *Spe*I-digested genomic DNA of the 146 isolates resulted in 126 reproducible and distinct PFPs or pulsotypes, with each profile consisting 13 to 23 DNA fragments ranging in size from 20 kb to 1100 kb (Figure 1). A wide genomic variation was evident among the strains and this is not surprising as the strains were recovered from sporadic cases of meliodosis. A cluster analysis of these 126 pulsotypes based on UPGMA algorithm produced a dendrogram (Figure 2). Based on the 85% similarity, 9 clusters (BS1-BS9) were observed. The largest cluster, BS9 (n=37) comprised of 22 pulsotypes. This was followed by BS3 (n = 20), BS7 (n = 18), BS5 (n =16) and BS4 (n=10) comprising of 19, 18, 15, 9 pulsotypes, respectively. The other pulsotypes represented less than 5% of the total and 27 (~18%) strains of *B. pseudomallei* did not fit into any of the 9 clusters.

The most common pulsotype BS9 was widely distributed and found in all the 10 states where isolates were obtained, but the next common type BS3 was detected only from the states of Kedah, Pahang, Penang, Perak and Sabah. Most states had 4 or more pulsotypes but Sarawak had only 3 types. BS1 was present in Kelantan and Pahang and BS2 only in Sarawak. The unclustered type was present in all states except Sabah and Sarawak (Table 1).

DISCUSSION

Genotyping by PFGE of the 146 *B. pseudomallei* clinical isolates resulted in 126 reproducible and distinct pulsotypes consisting of 13 to 23 banding patterns.

Chantratita *et al.* (2008) carried out PFGE on 600 soil isolates using *Spe*I restriction endonuclease that revealed 12 banding patterns. We are unable to explain this difference except these were soil isolates and from a different geographical location

Table 1. Distribution of *B. pseudomallei Spe* I-pulsotypes in Malaysia

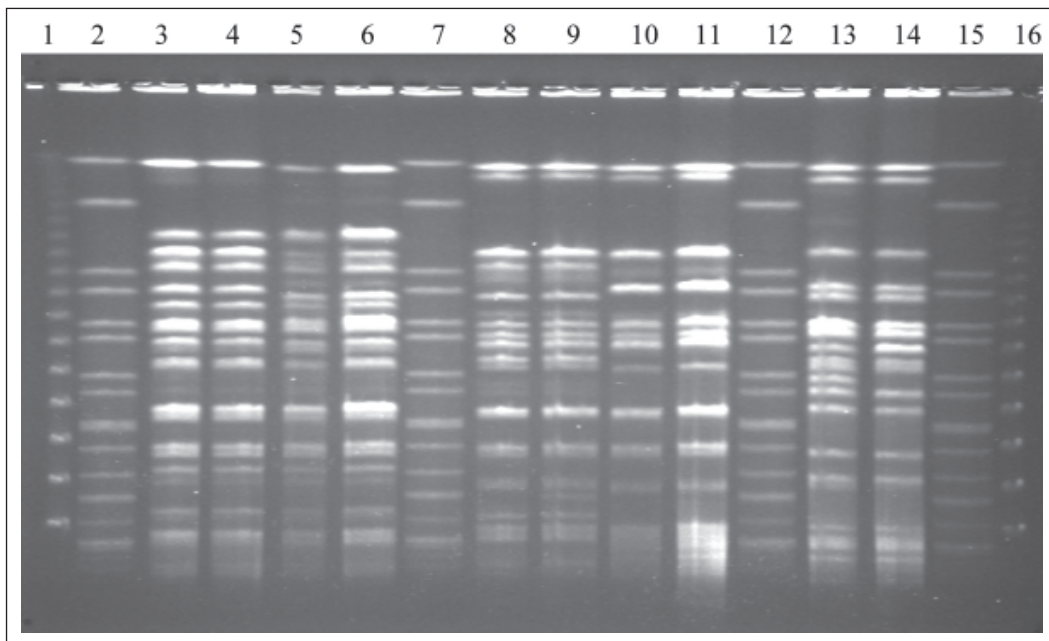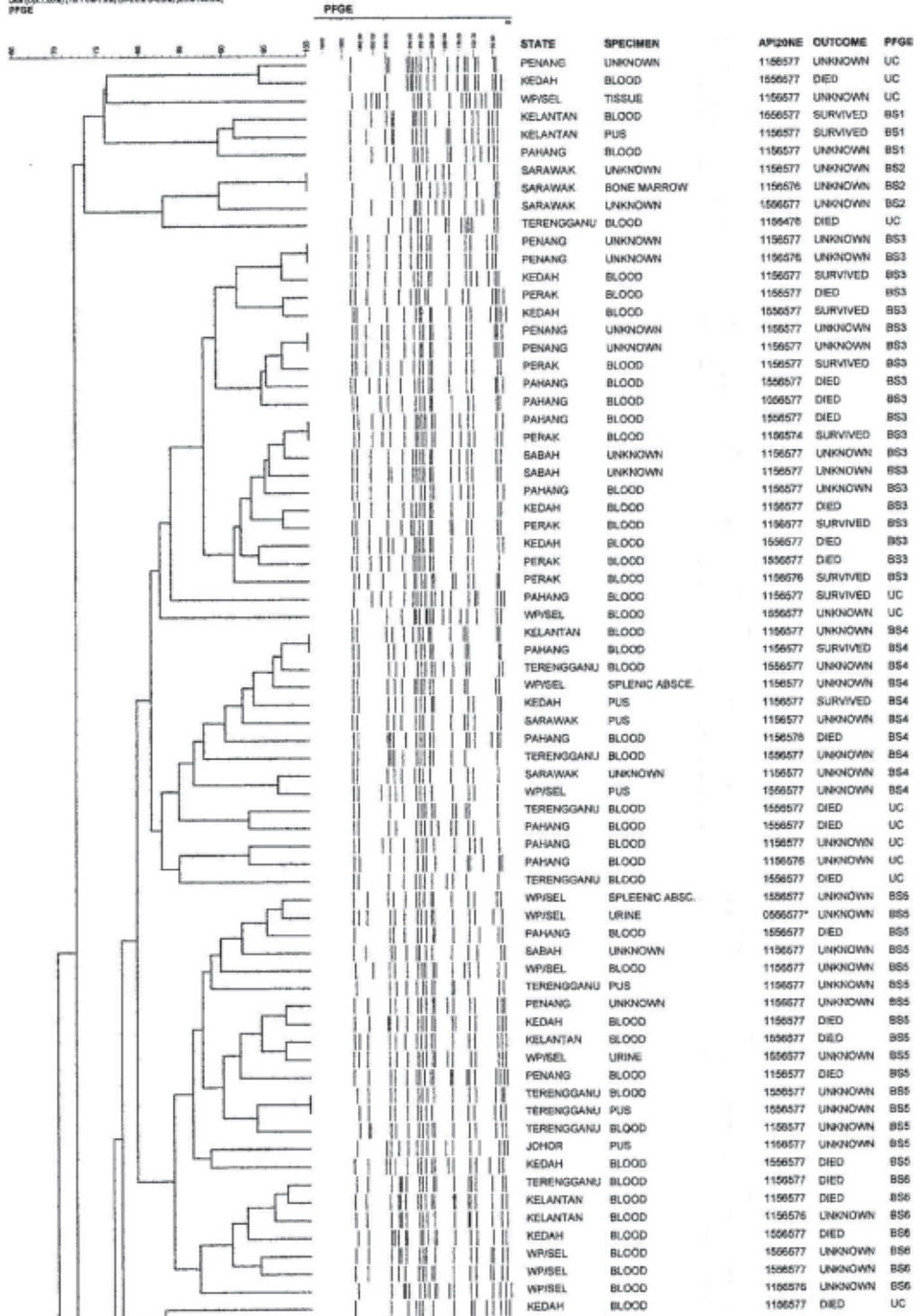| State/Pulsotype	BS1	BS2	BS3	BS4	BS5	BS6	BS7	BS8	BS9	UC	Total
Johor					1		4		1	1	7
Kedah			4	1	2	1			4	4	16
Kelantan	2			1	1	2		1	2	2	11
Pahang	1		4	2	1			1	3	6	18
Penang			4		2		1		3	1	11
Perak			6				3		4	1	14
Sabah			2		1			1	1		5
Sarawak		3		2					1		6
Terengganu				2	4	1	1		2	7	17
KL/Selangor				2	4	3	9	2	16	5	41
TOTAL	3	3	20	10	16	7	18	5	37	27	146

UC – unclustered

Figure 1. Optimum conditions of PFGE on *B. pseudomallei* at pulse time of 5 – 65 secs and runtime of 28 hours using *Spe*I restriction enzyme. Lanes 1 and 16: DNA Lambda concatamers, lanes 2, 7, 12, 15: *Salmonella* Braenderup H9812; Lane 3, 4, 5, 6, 8, 9, 10, 11, 13 and 14: different isolates of *B. pseudomallei* used for optimization

SPE1. (146 entries)

Dice (Opt:1.00%) (Tol 1.6%-1.6%) (H>0.0% S=0.0%) [0.0%-100.0%]
PFGE

PFGE

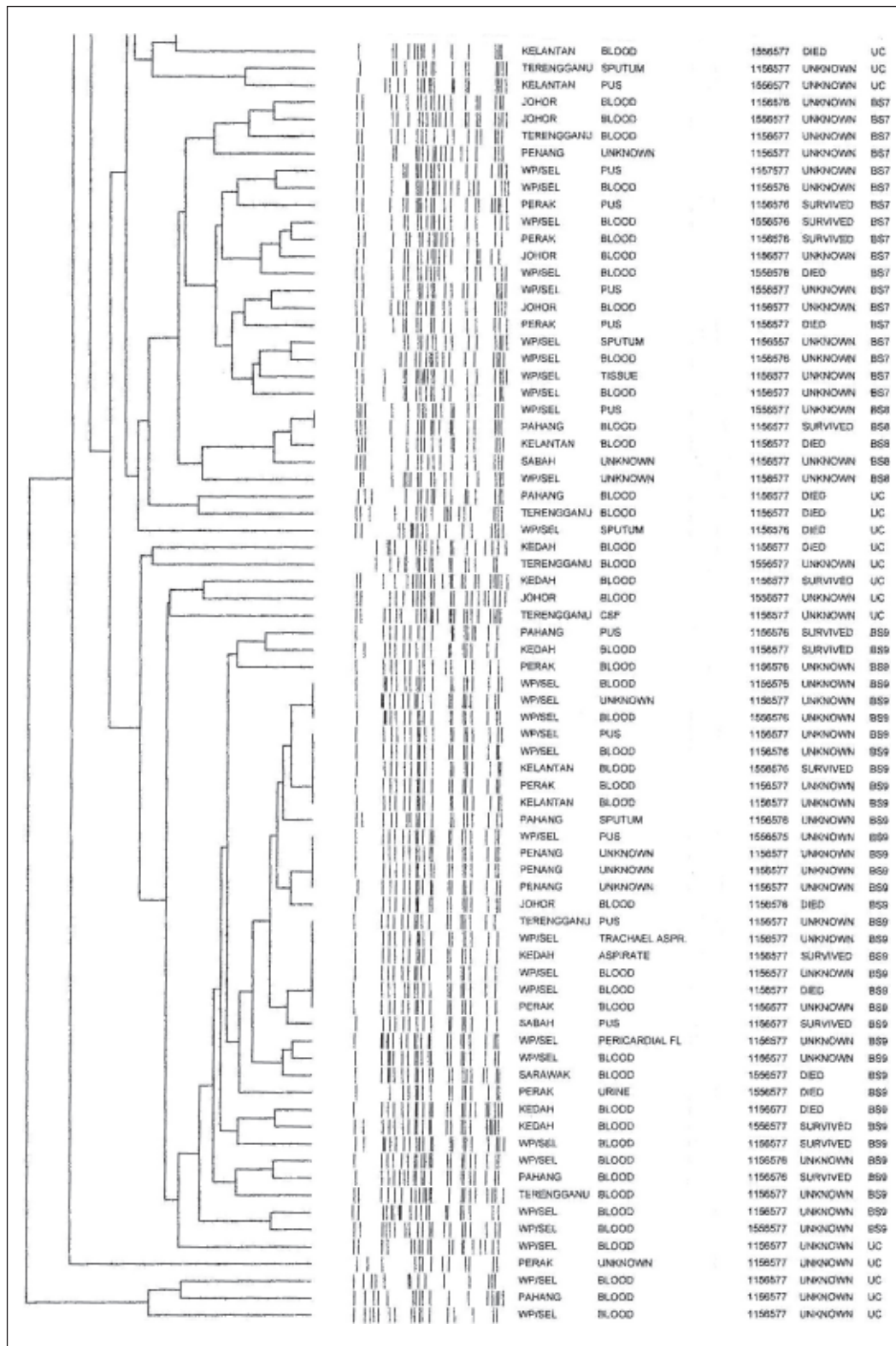| STATE	SPECIMEN	API20NE	OUTCOME	PFGE
PENANG	UNKNOWN	1156577	UNKNOWN	UC
KEDAH	BLOOD	1556577	DIED	UC
WP/SEL	TISSUE	1156577	UNKNOWN	UC
KELANTAN	BLOOD	1656577	SURVIVED	BS1
KELANTAN	PUS	1156577	SURVIVED	BS1
PAHANG	BLOOD	1156577	UNKNOWN	BS1
SARAWAK	UNKNOWN	1156577	UNKNOWN	BS2
SARAWAK	BONE MARROW	1156576	UNKNOWN	BS2
SARAWAK	UNKNOWN	1556577	UNKNOWN	BS2
TERENGGANU	BLOOD	1156476	DIED	UC
PENANG	UNKNOWN	1156577	UNKNOWN	BS3
PENANG	UNKNOWN	1196576	UNKNOWN	BS3
KEDAH	BLOOD	1156577	SURVIVED	BS3
PERAK	BLOOD	1156577	DIED	BS3
KEDAH	BLOOD	1556577	SURVIVED	BS3
PENANG	UNKNOWN	1156577	UNKNOWN	BS3
PENANG	UNKNOWN	1156577	UNKNOWN	BS3
PERAK	BLOOD	1156577	SURVIVED	BS3
PAHANG	BLOOD	1556577	DIED	BS3
PAHANG	BLOOD	1056577	DIED	BS3
PAHANG	BLOOD	1556577	DIED	BS3
PERAK	BLOOD	1156574	SURVIVED	BS3
SABAH	UNKNOWN	1156577	UNKNOWN	BS3
SABAH	UNKNOWN	1156577	UNKNOWN	BS3
PAHANG	BLOOD	1156577	UNKNOWN	BS3
KEDAH	BLOOD	1156577	DIED	BS3
PERAK	BLOOD	1156577	SURVIVED	BS3
KEDAH	BLOOD	1556577	DIED	BS3
PERAK	BLOOD	1556577	DIED	BS3
PERAK	BLOOD	1156576	SURVIVED	BS3
PAHANG	BLOOD	1156577	SURVIVED	UC
WP/SEL	BLOOD	1556577	UNKNOWN	UC
KELANTAN	BLOOD	1156577	UNKNOWN	BS4
PAHANG	BLOOD	1156577	SURVIVED	BS4
TERENGGANU	BLOOD	1556577	UNKNOWN	BS4
WP/SEL	SPLENIC ABSCE.	1156577	UNKNOWN	BS4
KEDAH	PUS	1156577	SURVIVED	BS4
SARAWAK	PUS	1156577	UNKNOWN	BS4
PAHANG	BLOOD	1156576	DIED	BS4
TERENGGANU	BLOOD	1556577	UNKNOWN	BS4
SARAWAK	UNKNOWN	1156577	UNKNOWN	BS4
WP/SEL	PUS	1556577	UNKNOWN	BS4
TERENGGANU	BLOOD	1556577	DIED	UC
PAHANG	BLOOD	1556577	DIED	UC
PAHANG	BLOOD	1156577	UNKNOWN	UC
PAHANG	BLOOD	1156576	UNKNOWN	UC
TERENGGANU	BLOOD	1556577	DIED	UC
WP/SEL	SPLEENIC ABSC.	1556577	UNKNOWN	BS5
WP/SEL	URINE	0556577*	UNKNOWN	BS5
PAHANG	BLOOD	1556577	DIED	BS5
SABAH	UNKNOWN	1156577	UNKNOWN	BS5
WP/SEL	BLOOD	1156577	UNKNOWN	BS5
TERENGGANU	PUS	1156577	UNKNOWN	BS5
PENANG	UNKNOWN	1156577	UNKNOWN	BS5
KEDAH	BLOOD	1156577	DIED	BS5
KELANTAN	BLOOD	1556577	DIED	BS5
WP/SEL	URINE	1556577	UNKNOWN	BS5
PENANG	BLOOD	1156577	DIED	BS5
TERENGGANU	BLOOD	1556577	UNKNOWN	BS5
TERENGGANU	PUS	1556577	UNKNOWN	BS5
TERENGGANU	BLOOD	1156577	UNKNOWN	BS5
JOHOR	PUS	1556577	UNKNOWN	BS5
KEDAH	BLOOD	1556577	DIED	BS5
TERENGGANU	BLOOD	1156577	DIED	BS6
KELANTAN	BLOOD	1156577	DIED	BS6
KELANTAN	BLOOD	1156576	UNKNOWN	BS6
KEDAH	BLOOD	1556577	DIED	BS6
WP/SEL	BLOOD	1556577	UNKNOWN	BS6
WP/SEL	BLOOD	1556577	UNKNOWN	BS6
WP/SEL	BLOOD	1156576	UNKNOWN	BS6
KEDAH	BLOOD	1156577	DIED	UC

Figure 2. Dendrogram generated from the PFGE profiles of *B. pseudomallei*

i.e. Thailand. Differences in the PFGE conditions especially the pulse times may have resulted in the different number of resolvable bands.

All our clinical isolates from the various states in Malaysia were found to be genetically diverse and heterogeneous. This is not surprising as the isolates were from sporadic cases and came from different sources and geographical locations. This is in keeping with published reports from other countries where *B. pseudomallei* isolates were found to be genetically diverse and heterogenous in macro restriction patterns (Koonpaew *et al.*, 2000; Pitt *et al.*, 2000; Wuthiekanun *et al.*, 2009). From Malaysia, Mohandas *et al.* (1994) and Vadivelu *et al.* (1997) published reports that were similar to the present study regarding heterogeneity of *B. pseudomallei* isolates but without reference to geographical locations regarding the source of the organisms. In contrast, Currie *et al.* (1994) found DNA from 8 animal isolates, a soil sample and one from a human case showed an identical ribotype on Southern blotting but this ribotype was different from the 3 commonest ribotypes seen in tropical Australia.

Based on the generated PFGE clusters by *Spe*I restriction enzyme, it was observed that clusters BS1, BS6 and BS7 were limited to the peninsular states of Malaysia whereas there were three BS 2 types isolated only from Sarawak, an east Malaysian state. Overall, there were genomic and molecular differences in the distribution of *B. pseudomallei* in the peninsular and east Malaysian states. At the same time, in particular, certain clusters of *B. pseudomallei* were present in some states and not in other states.

Cluster BS9 ($n = 37$) was widely distributed throughout peninsular Malaysia as well as in Kuala Lumpur, the populous capital city and in the state of Selangor, which also had the largest number of 41 isolates (Table 1). Within cluster BS9, some isolates from different states were indistinguishable, indicating the endemicity of this subtype in Malaysia. It is difficult to be absolutely sure that all these cases actually originated from this location due to the movement and migration of personnel in and out of this region. It is also noteworthy that although 41 isolates were from this particular location none belonged to Clusters BS1, BS2 and BS3. These 3 clusters were mainly from the rural areas of peninsular Malaysia and from Sabah and Sarawak which have been known as the hyperendemic states.

Although these 3 clusters appear to be present only in the rural and padi-growing regions of Malaysia, it is difficult to say categorically that they are from hyperendemic regions as the numbers of isolates were not sufficient for the calculation of statistical significance.

In conclusion, although PFGE typing was found to be highly discriminatory and reproducible for the epidemiological study of *B. pseudomallei*, and that some clusters were present in some states and not in others, overall, the study did not demonstrate any outstanding molecular differences between the hyper endemic and the normal endemic regions.

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