COMPARISON OF *PSEUDOMONAS PSEUDOMALLEI* FROM HUMANS, ANIMALS, SOIL AND WATER BY RESTRICTION ENDONUCLEASE ANALYSIS

E H Yap, T W Thong, A L Tan, M Yeo, H C Tan, H Loh, T P Teo, K T Thong, M Singh, Y C Chan

ABSTRACT

Pseudomonas pseudomallei* isolates from 62 human, 17 animal, 3 soil and 3 water samples were examined by genomic DNA digestion with PstI. Five major (RE I, II, III, IV, V) reproducible restriction patterns were observed, with most (56/62) of the human isolates displaying RE I (30/62), II (5/62), III (15/62), IV (4/62), V (2/62), and the animal (16/17), soil (2/3), water (3/3) isolates showing predominantly RE II profiles. Six human and one soil isolates showed patterns different from those of RE I to V. Restriction endonuclease analysis may be applied in epidemiological studies of melioidosis.

Keywords: Pseudomonas pseudomallei, restriction endonuclease analysis.

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INTRODUCTION

Melioidosis in man and animals is caused by *Pseudomonas pseudomallei* found in soil and water within endemic areas, predominantly in Southeast Asia and Northern Australia[1]. Infection is acquired by inoculation, inhalation or ingestion of these organisms. The most common symptomatic form of the disease is an acute pulmonary infection. This infection has the potential for latency, with recrudescence into an acute, often fulminating, and fatal infection[2]. In Singapore, there is an increasing number of melioidosis cases[3], and a possible aetiologic role of *P. pseudomallei* has been reported in Thai construction workers who succumbed to the sudden unexplained death syndrome (SUDS)[4].

Epizootic infections have been reported in sheep, goats and pigs living in the Caribbean islands[5] and in imported pigs in Singapore[6]. Other animals, like lamb[7], banded leaf-monkey[8], galah[9], horse, kangaroo[10] and dolphin[11] have died of melioidosis.

Typing methods for distinguishing between strains of *P. pseudomallei* are lacking. Dodin and Fournier[12] recognised two serotypes, I (Southeast Asia) and II (Australia), of *P. pseudomallei*. Plasmid typing is not possible since a low occurrence of plasmids has been observed in *P. pseudomallei* isolates[13]. In the present study, restriction endonuclease analysis (REA) of chromosomal DNA is described as a means of typing *P. pseudomallei*.

MATERIALS AND METHODS

Bacterial isolates

Eighty-five *P. pseudomallei* isolates were recovered from human, animal, soil and water sources. Sixty-two were isolated from cultures of blood, pus or sputum of human patients of different races from different hospitals in Singapore. Of the 17 animal isolates, 11 were from the Singapore Zoological Gardens (SZG), 3 from the Jurong Bird Park (JBP), 2 from local animal facilities and onc from a pet German Shepherd dog. Three water isolates were from moats within the animal enclosure in the zoo. Two soil isolates were from a southern island, off Singapore, and one from the compound of the house where the owners of the dog were residing.

For recovery of soil isolates, 100 g of soil was collected in 200 ml sterile distilled water. One ml of the suspension fluid was inoculated intraperitoneally into weanling hamsters. The hamsters were observed for 21 days. Post mortems were conducted on dead animals. Heart blood and pyogenic lesions were cultured onto MacConkey and blood agar plates.

All isolates were identified as *P. pseudomallei* based on colonial morphology and confirmed by biochemical tests using the API 20NE test kit. The isolates were maintained on tryptic soy agar slants and cultured monthly. Some isolates were maintained for a period of six months.

DNA extraction and restriction enzyme analysis

Bacterial isolates were grown in Trypticase soy broth at 37°C for 24 hours. 1.5 ml of culture was spun in a microcentrifuge for

Department of Microbiology
National University of Singapore
Kent Ridge Crescent
Singapore 0511

E H Yap, PhD
Associate Professor

T W Thong
Senior Laboratory Technologist

K T Thong
Laboratory Technologist

M Singh, PhD
Associate Professor

Y C Chan, PhD
Associate Professor

Department of Pathology
Singapore General Hospital
Outram Road
Singapore 0316

A L Tan, MBBS, FRCPA
Consultant

M Yeo, MBBS, DTM&H, DipBact, FRCPA, FAMS
Consultant

Central Veterinary Laboratory
Primary Production Department
2 Jalan Serangoon Kechil
Singapore 1954

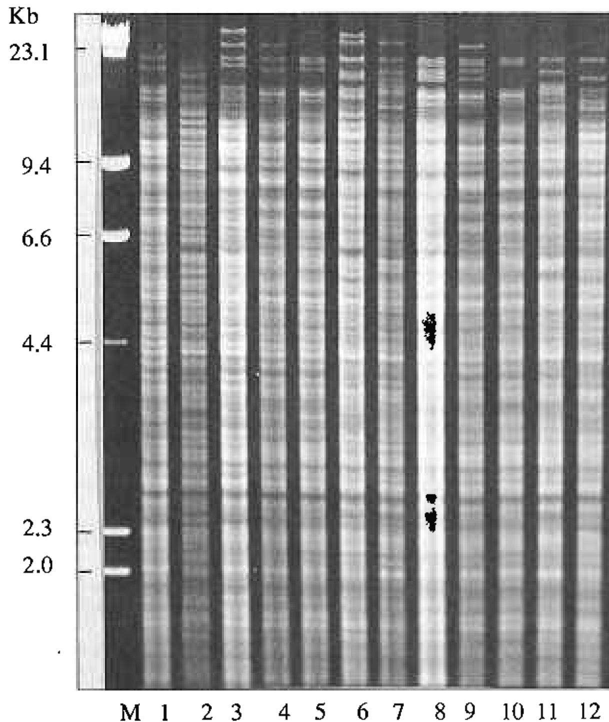
H C Tan, MSc
Bacteriologist

H Loh, B Vet Sc
Head

T P Teo
Senior Laboratory Technologist

Correspondence to: A/Prof E H Yap

Fig 1 – Restriction endonuclease (RE) patterns of *P. pseudomallei* after digestion with *Pst*I (10 units/ug DNA). Lambda DNA digested with *Hind*III was used as a size marker (lane M). The RE patterns are represented by lane 1 (RE I), lane 2 (RE II), lane 3 (RE III), lane 4 (RE IV), lane 5 (RE V) and lanes 6-12 (Others).



2 min and the pellet resuspended in 567 ul TE buffer (1 mM EDTA, 10 mM Tris.Cl, pH 8.0). 30 ul of 10% SDS and 3 ul of 20 mg/ml proteinase K were added to give a final concentration of 100 ug/ml proteinase K in 0.5% SDS. After mixing thoroughly, the mixture was incubated for 1 hour at 37°C. 100 ul of 5 M NaCl was added and the contents mixed before adding 80 ul of CTAB/NaCl solution. Following incubation for 10 min at 65°C, an equal volume of chloroform/isoamyl alcohol (24:1) was added, and after mixing, the tube was spun for 4 to 5 min in a microcentrifuge. The aqueous, viscous supernatant was transferred to a fresh microcentrifuge tube and an equal volume of 24:24:1 phenol/chloroform/isoamyl alcohol was added, before spinning the extract for 5 min. 0.6 vol isopropanol was added to the transferred supernatant, and the resultant precipitate pelleted by spinning briefly at room temperature before washing with 70% ethanol. The pellet was redissolved in 100 ul TE buffer. The final DNA concentration was determined using a spectrophotometer, with an optical density at 260 nm of 1 being equal to 50 ug of DNA per ml.

For restriction endonuclease digestion, 5 ug of purified DNA was incubated at 37°C for 1 to 2 hours with 50 units of *Pst*I (Boehringer Mannheim, Germany) in a reaction mixture using buffer supplied by the manufacturer. DNA fragments were separated by electrophoresis in a 0.6% agarose (FMC SeaKem LE) gel on a horizontal gel bed (250 by 200 mm) with Tris-borate-EDTA (89 mM Tris borate, 2 mM EDTA) as the running buffer. After electrophoresis at 60 V for 16 hours, the gels were stained for 1 hour with ethidium bromide (0.125 mg/ml) before photography.

RESULTS

Preliminary digestion of DNA from *P. pseudomallei* with different restriction enzymes produced varying results, with *Pst*I giving clear and reproducible bands, particularly with DNA fragments of about 10 to 30 kilobases pairs (Fig 1). Digestion with *PstI* yielded 5 major restriction profiles, RE I, II, III, IV and V, with 7 other isolates showing unique profiles (Fig 1). The bands were reproducible even after subculturing for a period of six months. Most of the human isolates showed RE I and III patterns, fewer of RE II, IV and V (Table I). Although most of the patients lived in different areas within the 650 sq km island of Singapore, there was no apparent clustering of *P. pseudomallei* types in any particular area, nor a predominance of RE types amongst the organisms recovered from Chinese, Malay or Indian patients (data not shown).

Table I – Distribution of restriction endonuclease (RE) profiles among the *P. pseudomallei* isolates

RE Group	No. of isolates			
	Human	Animal	Soil	Water
I	30	1	–	–
II	5	16	2	3
III	15	–	–	–
IV	4	–	–	–
V	2	–	–	–
Others	6	–	1	–
Total	62	17	3	3

RE I isolates were recovered from the 2 patients staying in the island off Singapore but the soil isolates from this locale were RE II and one with a unique profile (Fig 1, lane 12).

In contrast, most of the animal isolates were of the RE II, with only one isolate displaying the RE I type profile. The latter was isolated from a crown pigeon from the JBP. Similarly, the soil and water isolates were mostly of RE II, with one soil isolate showing a unique profile.

The *P. pseudomallei* from the 4 gorillas, and the water from the SZG belonged to the RE II group. Similarly the same RE II types were recovered from the German Shepherd dog as well as the soil sample from where it lived.

DISCUSSION

To date, only two serotypes, I and II, of *P. pseudomallei* have been described[12]. Recently, two groups of workers reported using ribotyping which was capable of discriminating up to 22 strains of *P. pseudomallei*[14,15]. The present study confirms that these organisms may be divided into at least 12 RE types, with most of them clustered into 5 larger groups, namely RE I to V.

The REA was applied to three epidemiological situations. The first involved two patients who died of melioidosis whilst staying in an island off Singapore. Soil samples from the area where they lived were cultured for *P. pseudomallei*. Whilst the isolates recovered from the patients belonged to the RE I group, those from the soil displayed RE II and another showed a profile different from RE I to V. It was possible they may have acquired the infection from organisms in other areas.

The second situation involved four gorillas, originally from Europe where they had been housed in a concrete environment for most of their lives before they were sent to the SZG. They died of melioidosis within months of living in an 'open' enclosure where they were unwittingly exposed to soil and water (in the surrounding moat) containing *P. pseudomallei*. Isolates from the gorillas were all of the RE II group, similar to the isolates

recovered from the water of the moat, implying that the animals acquired the infection from organisms in the water. It is interesting to note that isolates from the other animals who died in the same zoo, namely gibbon, kangaroo, mandrill and chimpanzee, were of the RE II group. Isolates from birds belonging to the Jurong Bird Park, located several kilometers away from the SZG, were also of the RE II (cassowary and palm cockatoo) as well as RE I (crown pigeon) types.

In the third situation, an imported German Shepherd dog died of melioidosis. Although there was no evidence of external injuries, pneumonia in the right pulmonary lobes was evident and an abscess was found in the pons area of the right brain. The blood-stained purulent contents of the abscess yielded a pure culture of *P. pseudomallei* belonging to the RE II group. One out of 4 soil samples collected from various locations of the compound in the house where the dog lived yielded *P. pseudomallei*, also of the RE II group. It was likely that the dog was infected with organisms prevalent in the soil of the house. The incidence of melioidosis in imported animals may be greater than hitherto realised since they may succumb due to a lack of immunity, particularly in areas where *P. pseudomallei* is not prevalent, as in Europe where the German Shepherd and the gorillas had originally lived.

The above results suggest that there are many RE types of *P. pseudomallei* infecting both man and animals although RE I and III are predominant in man whilst RE II is more common in animals. Transmission from animals to man has not been documented and only one case of man-to-man transmission by a venereal route has been reported[16]. Although the owners were in close contact with their pet German Shepherd dog, none of them had detectable serum antibodies to *P. pseudomallei* as assessed by the indirect haemagglutination test[17].

Although the number of soil and water-derived *P. pseudomallei* is small and sampling biased, it may be worthwhile to identify the source of RE I and III types predominantly infecting humans.

Our data indicate that REA may be used as a reliable means of discriminating between different strains of *P. pseudomallei* which may prove useful for typing these organisms in epidemiologic studies. It is possible that the *P. pseudomallei* may be further subtyped. Work is in progress to apply the pulsed field gel electrophoresis method which had proven to be highly successful in the comparison of genomic DNA of *Enterococcus faecalis*[18], *Acinetobacter calcoaceticus, P. aeruginosa*[19] and *Listeria monocytogenes*[20].

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