

***Pseudomonas pseudomallei* isolates collected over 25 years from a non-tropical endemic focus show clonality on the basis of ribotyping**

B. CURRIE¹, H. SMITH-VAUGHAN¹, C. GOLLEDGE², N. BULLER³,
K. S. SRIPRAKASH¹ AND D. J. KEMP¹

¹*Menzies School of Health Research and Royal Darwin Hospital, Darwin, Australia*

²*Sir Charles Gairdner Hospital, Perth, Australia*

³*Western Australian Department of Agriculture, Perth, Australia*

(Accepted 24 April 1994)

SUMMARY

Between 1966 and 1991, melioidosis, a disease caused by *Pseudomonas pseudomallei* that is mostly confined to tropical regions, occurred in farm animals and a farmer in temperate south-west Western Australia. Using an *Escherichia coli* probe containing a ribosomal RNA operon, *P. pseudomallei* DNA from isolates from 8 animals, a soil sample and the human case showed an identical ribotype on Southern blotting. The ribotype was different from the 3 commonest ribotypes seen in tropical Australia. This molecular typing supports the theory of clonal introduction of *P. pseudomallei* into a non-endemic region, with environmental contamination, local dissemination and persistence over 25 years. As melioidosis is often fatal in humans, such persistence in a temperate region is cause for concern.

INTRODUCTION

Melioidosis is a disease of animals and humans caused by *Pseudomonas pseudomallei* and the major endemic foci are Southeast Asia and tropical Australia [1, 2]. Infection in Australia was first diagnosed in sheep in 1949 [3] and in a human in 1950 [4], both occurring in north Queensland. *P. pseudomallei* is now the commonest organism isolated in fatal community-acquired pneumonia in the tropical region of the Northern Territory of Australia [5].

Since 1966, cases of melioidosis have been described in farm animals in temperate south-west Western Australia, 50–250 km north of Perth [6, 7], and in 1991 a hobby farmer from the region (latitude 31° 10' S) presented with fever and a mediastinal mass from which *P. pseudomallei* was cultured [7]. It was suggested that melioidosis was introduced into the farms by imported carrier animals from tropical Australia, or alternatively that the organism had survived in the soil from ancient times when rain forest covered the area [5–7]. Using ribotyping we have

* Correspondence and reprints: Dr Bart Currie, Menzies School of Health Research, P.O. Box 41096, Casuarina NT 0811, Australia.

confirmed the clonality of *P. pseudomallei* isolated between 1966 and 1991 from local animals, soil and the human case.

METHODS

Stored freeze dried isolates were cultured on blood agar and *P. pseudomallei* identity was confirmed biochemically by API 20E (Biomérieux). In addition 3 cultures from melioidosis cases at Royal Darwin Hospital, Northern Territory, were included. These represented the 3 commonest ribotypes found in isolates from the Northern Territory [8, and unpublished data].

DNA preparation

Total chromosomal DNA was extracted [9]. A 1.5 ml overnight cell culture was pelleted by centrifugation. Pelleted cells were washed twice in 10 mM Tris-HCl 1 mM EDTA pH8 (TE). RNase was added to 200 μ l of the resuspended pellet at 150 μ g/ml and incubated at 37 °C for 30 min. To this, proteinase K (100 μ g/ml) was added and incubated at 65 °C for 2 h, followed by 500 μ l 5 M guanidinium thiocyanate, 100 mM EDTA and 0.5% v/v sarkosyl. This mixture was placed on ice and 250 μ l 7.5 M ammonium acetate was added. After extraction once with chloroform/isoamyl alcohol the DNA was precipitated with isopropanol (0.54 volume). Following centrifugation, the pellet was washed 3 times with 70% ethanol and resuspended in TE.

Restriction enzyme digestion

DNA was digested with *Bam*H1 (Pharmacia) as per the manufacturer's instructions.

Gel electrophoresis and Southern transfer

DNA restriction fragments were separated by electrophoresis on a 0.8% agarose gel and transferred to a positively charged membrane (hybond N+, Amersham) in 0.4 M NaOH.

rDNA probe

The probe was prepared by random primer labelling with [α -³²P]dATP [10] of the insert from plasmid pKK3535 [11], containing an *E. coli* ribosomal RNA operon.

DNA hybridization

The blot was pre-hybridized for 4 h at 65 °C before addition of the labelled rDNA probe. The pre-hybridization mixture contained 10% dextran sulphate, 4 \times standard saline citrate (SSC), 5 \times Denhardt's mix, 100 μ g/ml sheared herring sperm DNA and 50 mM sodium phosphate buffer (pH 7). Hybridization was for 16 h at 65 °C and post-hybridization washes were at 65 °C with 0.1% SDS/1 \times SSC. Autoradiograms of the patterns from hybridization were then compared.

RESULTS

Figure 1 shows geographical locations of the isolates. Hybridization patterns are shown in Figure 2, with lane identity in Table 1. The 10 isolates from the

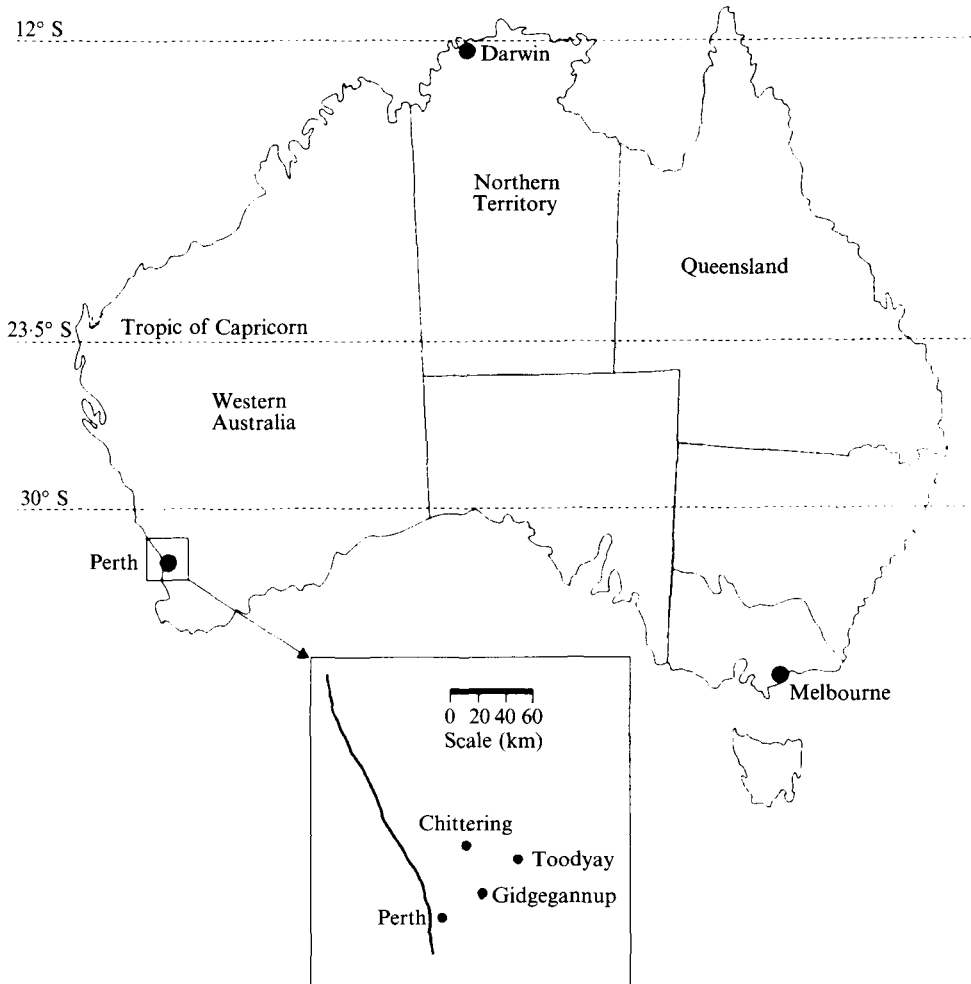


Fig. 1. The temperate area endemic for melioidosis.

temperate region were identical, while the 3 Darwin isolates were each different from the temperate isolates and also different from each other.

DISCUSSION

Our results support the clonal introduction of *Pseudomonas pseudomallei* into a non-tropical region of Australia, with subsequent persistence and local dissemination of the organism over 25 years. Although the definition of a clone using molecular typing awaits standardization, the pattern of the 10 identical Southern blots from the temperate region (identical to ribotype 15, reference 8) has yet to be seen in isolates from elsewhere in Australia. This is in contrast to the ribotypes in lanes 1–3, which were amongst the commonest found of the 18 ribotypes described from tropical Australia [8], and which together account for over 60% of Northern Territory human isolates [A. Lew, P. Desmarchelier, B. Currie, unpublished data]. Repeated isolation of the same ribotype over 25 years suggests

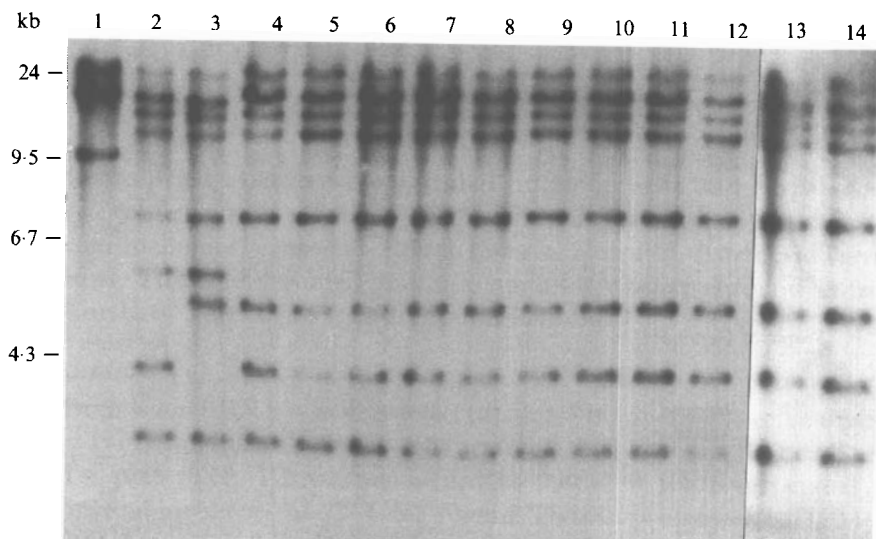


Fig. 2. Southern blots of *Pseudomonas pseudomallei* isolates. Procedures were as described in Materials and Methods. The samples run in lanes 1–14 are described in Table 1. Those in lanes 13 and 14 were in a different gel to those in lanes 1–12, but lane 14 is a repeat of the same sample as in lane 12.

Table 1. Source, location and year of the *Pseudomonas pseudomallei* isolates

Lane	Source	Location	Year
1	Human	Darwin	1991
2	Human	Darwin	1990
3	Human	Darwin	1991
4	Sheep	Chittering	1966
5	Sheep	Chittering	1966
6	Sheep	Chittering	1968
7	Sheep	Chittering	1978
8	Sheep	Chittering	1980
9	Soil	Chittering	1980
10	Goat	Gidgegannup	1983
11	Dog	Gidgegannup	1985
12 and 14	Goat	Gidgegannup	1987
13	Human	Toodyay	1991

environmental stability of the ribotype and our data clearly show that this ribotype has broad host specificity which includes humans.

The epidemiology of melioidosis in the temperate region is described in detail elsewhere [7]. It is thought that an infected animal imported from tropical Australia, possibly a dog or horse [6], introduced the organism, which subsequently survived in soil [2] and infected local animals including sheep, goats, horses and a dog [6, 7]. Movement of animals between farms is likely to account for the local spread of melioidosis over an area of 200 km long. In 1990 the farmer who subsequently contracted melioidosis bought a lamb, which later died of pneumonia of unidentified cause. It is most likely that the farmer acquired melioidosis percutaneously from contaminated soil, although inhalation or direct animal contact are also possible [1, 2, 5]. That such contamination can persist for 25 years in a temperate region is cause for concern.

A number of outbreaks of melioidosis in animals have been attributed to imported infection, including sheep, goats and pigs on Aruba in the Netherlands Antilles [12], horses in Iran and France [2, 13] and cattle in south-eastern Queensland [14]. The French outbreak in the 1970s, which occurred in zoos, equestrian clubs and racecourses, resulted in fatal cases of melioidosis in animal handlers and extensive environmental contamination lasting some years [2].

More recently, concern that transmission to other animals, animal handlers and the environment might occur resulted in euthanasia of many infected cynomolgus monkeys imported into Britain from the Philippines [15]. Investigations revealed infections in epidemiologically unrelated monkeys from Indonesia, reflecting the increasing recognition of *P. pseudomallei* as an important pathogen of both animals and humans [5, 15].

Ribotyping has been used to demonstrate the clonality of initial and subsequent isolates in relapsed melioidosis [16], and our findings support the ribotype as a stable epidemiological marker of *P. pseudomallei*, with an identical pattern in related isolates spanning 25 years. Further molecular studies using methods such as ribotyping will help elucidate the complex epidemiology of melioidosis in both endemic regions and areas with newly recognized infection.

ACKNOWLEDGEMENTS

The plasmid used for probing was kindly provided by Dr Bruce Ross of the Royal Children's Hospital, Melbourne, Australia. We would like to thank Gary Myers, Kay Withnall and Dae Sharrock for technical support, and Bee Fong Gunn and Jill Albion for help preparing the manuscript. Earlier ribotyping of Darwin isolates was established by Patricia Desmarchelier and Ala Lew.

REFERENCES

1. Leelarasamee A, Bovornkitt S. Melioidosis: review and update. *Rev Infect Dis* 1989; **11**: 413–25.
2. Dance DAB. Melioidosis: the tip of the iceberg? *Clin Microbiol Rev* 1991; **4**: 52–60.
3. Cottew GS. Melioidosis in sheep in Queensland. A description of the casual organism. *Aust J Exp Biol Med Sci* 1950; **28**: 677–83.
4. Rimington RA. Melioidosis in north Queensland. *Med J Aust* 1962; **1**: 50–3.
5. Currie BJ. Medicine in tropical Australia. *Med J Aust* 1993; **158**: 609–15.
6. Ketterer PJ, Bamford VW. A case of melioidosis in lambs in south Western Australia. *Aust Vet J* 1967; **43**: 79–80.
7. Golledge CL, Chin WS, Tribe AE, Condon RJ, Ashdown LR. A case of human melioidosis originating in south-west Western Australia. *Med J Aust* 1992; **157**: 332–4.
8. Lew AE, Desmarchelier PM. Molecular typing of *Pseudomonas pseudomallei*: restriction fragment length polymorphisms of rRNA genes. *J Clin Microbiol* 1993; **31**: 533–9.
9. Pitcher DG, Saunders NA, Owen RJ. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* 1989; **8**: 151–6.
10. Feinberg AP, Vogelstein B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 1983; **132**: 6–13.
11. Altwegg M, Hickman-Brenner FW, Farmer JJ. Ribosomal RNA gene restriction patterns provide increased sensitivity for typing *Salmonella typhi* strains. *J Infect Dis* 1989; **160**: 145–9.
12. Suttmoller P, Kraneveld FC, van der Schaaf A. Melioidosis (*Pseudomalleus*) in sheep, goats and pigs on Aruba Netherlands Antilles. *J Am Vet Med Assoc* 1957; **130**: 415–7.

13. Mollaret HH. 'L'affaire du Jardin des plantes' ou comment la melioidose fit son apparition en France. *Med Mal Infect* 1988; **18**: 643–54.
14. Ketterer PJ, Donald B, Rogers RJ. Bovine melioidosis in south-eastern Queensland. *Aust Vet J* 1975; **51**: 395–8.
15. Dance DAB, King C, Aucken H, Knott CD, West PG, Pitt TL. An outbreak of melioidosis in imported primates in Britain. *Vet Rec* 1992; **130**: 525–9.
16. Desmarchelier PM, Dance DAB, Chaowagul W, Suputtamongkol Y, White NJ, Pitt TL. Relationships among *Pseudomonas pseudomallei* isolates from patients with recurrent melioidosis. *J Clin Microbiol* 1993; **31**: 1592–6.