Fine-Scale Genetic Diversity among *Burkholderia pseudomallei* Soil Isolates in Northeast Thailand[∇]

Jana M. U'Ren,[1] Heidie Hornstra,[1] Talima Pearson,[1] James M. Schupp,[1] Benjamin Leadem,[1]
Shalamar Georgia,[1] Rasana W. Sermswan,[2] and Paul Keim[1]*

Northern Arizona University, Center for Microbial Genetics and Genomics, Flagstaff, Arizona 86011,[1] and
Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand[2]

***Burkholderia pseudomallei* soil isolates from northeast Thailand were genotyped using multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA) and multilocus sequence typing (MLST). MLVA identified 19 genotypes within three clades, while MLST revealed two genotypes. These close genetic relationships imply a recent colonization followed by localized expansion, similar to what occurs in an outbreak situation.**

Burkholderia pseudomallei is the causative agent of melioidosis, a potentially fatal disease endemic in areas of northern Australia and southeast Asia (10, 19). In the northeast of Thailand, melioidosis is accountable for 20% of community-acquired septicemias and is associated with a 40% mortality rate (2). Melioidosis is acquired by skin exposure, inhalation, or ingestion of the pathogen (19). Thus, commonly infected Thai individuals are those that encounter soil and surface water contaminated with *B. pseudomallei*, especially rice farmers (13). While this disease is exotic and rare in much of the world, it is a major public health problem in Thailand.

As expected from such high incidences of melioidosis, *B. pseudomallei* is widely distributed and commonly isolated from soil and water throughout areas of endemicity (6, 17, 18). Molecular analyses of the genetic diversity of clinical and environmental *B. pseudomallei* isolates have revealed that at various geographic and temporal scales, *B. pseudomallei* has substantial genetic diversity (7, 9, 16, 17). However, few studies have examined the soil reservoir at defined geographic and temporal scales to assess whether *B. pseudomallei* genetic diversity is representative of regional diversity or alternatively demonstrates lower levels of diversity. To examine this question, we assessed the genetic diversity and spatial distribution of *B. pseudomallei* in the soil from a 50-km² region of Khon Kaen Province, Thailand, during a 6-month time span in 2004.

In this study, soils were collected from 50 sites along both sides of intervillage roads. Sampling locations were recorded using a Global Positioning System receiver and mapped using ArcGIS 9.1 software. At each site, three holes, positioned in a triangle, were dug using a 5-inch-diameter hand auger. Three grams of soil was collected from three depths in each hole (15, 30, and 45 cm). Soil was vortexed with 3 ml distilled water for 30 s, followed by a settling time of 30 min. Five hundred microliters of supernatant was transferred into 3 ml selective enrichment broth (threonine-basal salt solution with 20 μg/liter of colistin) (20) and incubated at 42°C for 48 h with

shaking. One hundred microliters of broth was 10-fold serially diluted to concentrations of 10^{-2}, 10^{-3}, and 10^{-4}, and 10 μl of each dilution was cultured on Ashdown's agar (1), incubated at 42°C for 4 to 7 days, and examined daily for emerging colonies. Presumptive *B. pseudomallei* colonies were confirmed using biochemical tests, including L-arabinose assimilation.

Nineteen of the 50 sites were culture positive for *B. pseudomallei*. From those 19 sites, 68 *B. pseudomallei* colonies were cultured, and total genomic DNA was extracted by a phenol-chloroform technique (11) from single colonies. In addition, from five culture-positive sites, eight DNA samples (Table 1) were extracted directly from 2 g of soil using a bead beating (5) and phenol-chloroform protocol (21). DNA was then purified to remove inhibitors by agarose gel extraction (22).

Before beginning genetic characterization, the 68 isolates were confirmed to be *B. pseudomallei* with a species-specific real-time PCR assay (15). For genetic characterization, 26 loci from a previously described multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA) system were used to genotype the 68 cultured isolates as well as the eight DNAs extracted directly from the soil (16). DNA was also genotyped by a ribotyping method using HindIII enzyme for digestion and 16S and 23S rRNA genes of *Escherichia coli* as a probe (12). Further genetic analysis of isolates that were representative of each MLVA genotype was accomplished using multilocus sequence typing (MLST) (Table 1) (7), with the exception of genotype 10, which could not be sequenced. Sequences for each locus were queried against the online MLST database (http://bpseudomallei.mlst.net) to determine allelic designations and a subsequent allelic profile query was used to determine the sequence type (ST).

The degree of VNTR variability was assessed by the number of alleles observed and by Nei's diversity index: $D = 1 - \sum(\text{allele frequency})^2$. A Mantel test was performed to test the correlation between genetic distance and geographic distance (8). An unrooted distance-based phylogenetic tree and mean pairwise genetic diversity were calculated for representatives of each genotype from 26 VNTR loci using the neighbor-joining algorithm in PAUP 4.0 b10 (14).

In the collection of *B. pseudomallei* isolates, 23 VNTR loci

---

* Corresponding author. Mailing address: Northern Arizona University, Department of Biological Sciences, Box 5640, Flagstaff, AZ 86011-5640. Phone: (928) 523-1078. Fax: (928) 523-0639. E-mail: Paul.Keim@nau.edu.

TABLE 1. Location, depth, and genotypes for the 68 isolates of *B. pseudomallei*

Isolate name	Sample site	Depth (cm)	Ribotype	ST by MLST	MLVA genotype
001[a]	2	15	23	70	A1
002[a]	2	15	23		A1
003	2	30	23		A1
005	2	30	23		A1
009	8	30	23		A1
010	8	30	23		A1
011	10	15	16		A1
014	11	30	23		A1
016	11	30	23		A1
018[a]	13	30	23	70	A2
004[a]	2	30	23	70	A3
008	8	30	23		A4
012	10	30	23		A4
013	11	30	23		A4
19	13	30	23		A4
020[a]	15	15	NA[c]		A4
024	22	15	16		A4
025	22	30	23		A4
026	22	30	23		A4
028	22	30	23		A4
029	24	15	23		A4
030[a]	24	30	23		A4
031	24	30	23		A4
032[a]	24	30	23		A4
033	50	15	23		A4
034	50	30	23		A4
036	50	45	23		A4
037[a]	26	30	NA		A4
038	26	30	23	70	A4
039	26	45	23		A4
040	27	15	23		A4
041	27	15	23		A4
042	27	30	23		A4
046	29	30	23		A4
047	29	30	23		A4
048	29	30	23		A4
049	30	15	23		A4
052	30	30	23		A4
053	31	30	23		A4
054	31	30	23		A4
055	36	15	23		A4
056	36	30	23		A4
058	36	30	23		A4
059	36	30	23		A4
060	38	30	23		A4
061	38	45	23		A4
062	41	15	23		A4
063	41	15	23		A4
066	38	30	23		A4
035	50	30	23	70	A5
023	15	30	23	70	A6
015	11	30	23	70	A7
045	29	15	23	70	A8
050	30	15	23	70	A9
064	45	30	23	NA	A10
017	13	15	23	70	A11
068	45	15	23		A11
057	36	30	23	70	A12
027	22	30	23	70	A13
051	30	30	23	70	A14
067	44	30	23	70	A15
007	8	15	13	70	B16
021	15	30	13		B16
022	15	30	13		B16
006	8	15	21		C17
065	38	15	21	NA[b]	C17
043	27	30	21	NA[b]	C18
044	29	15	21	NA[b]	C19

[a] DNA also extracted directly from soil.
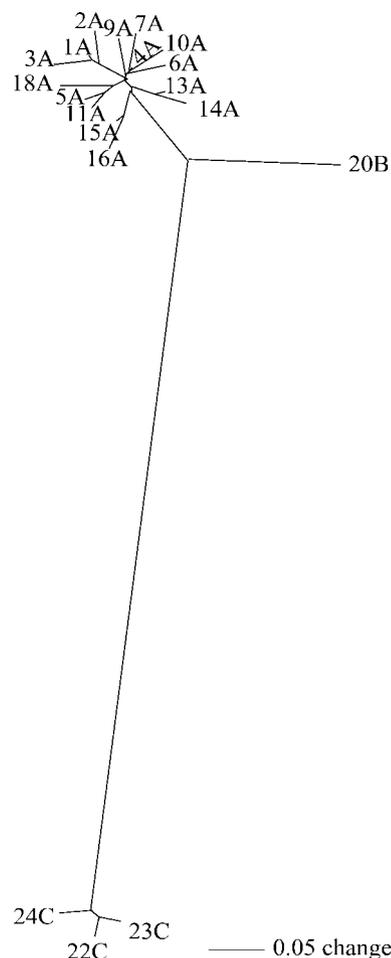[b] Novel ST (alleles 3, 1, 3, 2, 5, 2, 1).
[c] NA, not available.

FIG. 1. Unrooted neighbor-joining phylogram of 68 *B. pseudomallei* isolates. Numbers on the branches indicate the genotype, and A, B and C indicate the three genetic clades.

were polymorphic while 3 were monomorphic. For polymorphic loci, the number of alleles ranged from one to five, with Nei's diversity values ranging from 0.11 to 0.29. Phylogenetic analysis of MLVA data identified 19 genotypes within three clades (A, B, and C) (Fig. 1). Sixty-one of the isolates belonged to clade A, which was dominated by a single genotype (A4) (n = 38; 56%). Clade B contained three isolates of the same genotype. Clade C contained four isolates and three genotypes (Fig. 1). The mean pairwise genetic distance for the 19 genotypes was 0.16, which is lower than the calculated distance for a similarly localized *B. pseudomallei* population in northern Australia, even when considering only isolates from the same year (distance = 0.289) (9).

The genetic groups defined by MLVA were also confirmed by other typing methods (Table 1). For example, MLST revealed two STs for representatives of the 19 MLVA genotypes (ST70 for clades A and B and a novel ST for clade C). The novel ST is a single-locus variant to ST173 where the *narK* locus contains allele 2 instead of allele 4. Ribotyping identified four types (R16 and R23 for clade A, R13 for clade B, and R21 for clade C) which mirror the genetic relationship revealed by MLVA (Table 2). Additionally, a phylogenetic comparison of

TABLE 2. Ribotype patterns of *B. pseudomallei* isolates

MLVA clade	Ribotype	Presence of ribotype fragment[a]:															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
A	16	1	0	0	1	0	0	0	**0**	0	1	**1**	0	0	**1**	0	0	1
A	23	1	0	0	1	0	0	0	**0**	0	1	**0**	0	0	**1**	0	0	1
B	13	1	0	0	1	0	0	**1**	0	1	0	**1**	0	0	**1**	0	**1**	1
C	21	1	0	0	1	0	0	**1**	0	1	0	**1**	0	0	**0**	**1**	**1**	**0**

[a] Differences in the ribotype pattern are highlighted in boldface. 1, present; 0, absent.

all 212 Thai MLST types (obtained from the online database www.bpseudomalleimlst.net) reveals that the STs found during this study are distantly related, therefore, supporting the genetic distance of MLVA clades A and B from clade C. Thus, the overall pattern of three typing methods demonstrates lim-

ited genetic diversity across the sampled region and the separation of isolates into a small number of phylogenetic groups.

The effects of culture bias could potentially influence which genotypes were observed; hence, a preliminary analysis of the eight DNA isolates extracted directly from soil, which represent five culture-positive soil sites, was used to confirm the presence of genotypes found through culturing. Three of the soil isolates (all from site 2) failed to amplify across all loci, most likely due to the crude purification method used. Additionally, multiple minor peaks were observed for some loci, suggesting the presence of mixed genotypes. However the dominant allele peaks matched clade A alleles from cultures obtained at the same sites. This preliminary evidence suggests that culture-dependent techniques recover the dominant *B. pseudomallei* genotypes, although the presence of other low-frequency genotypes cannot be excluded.
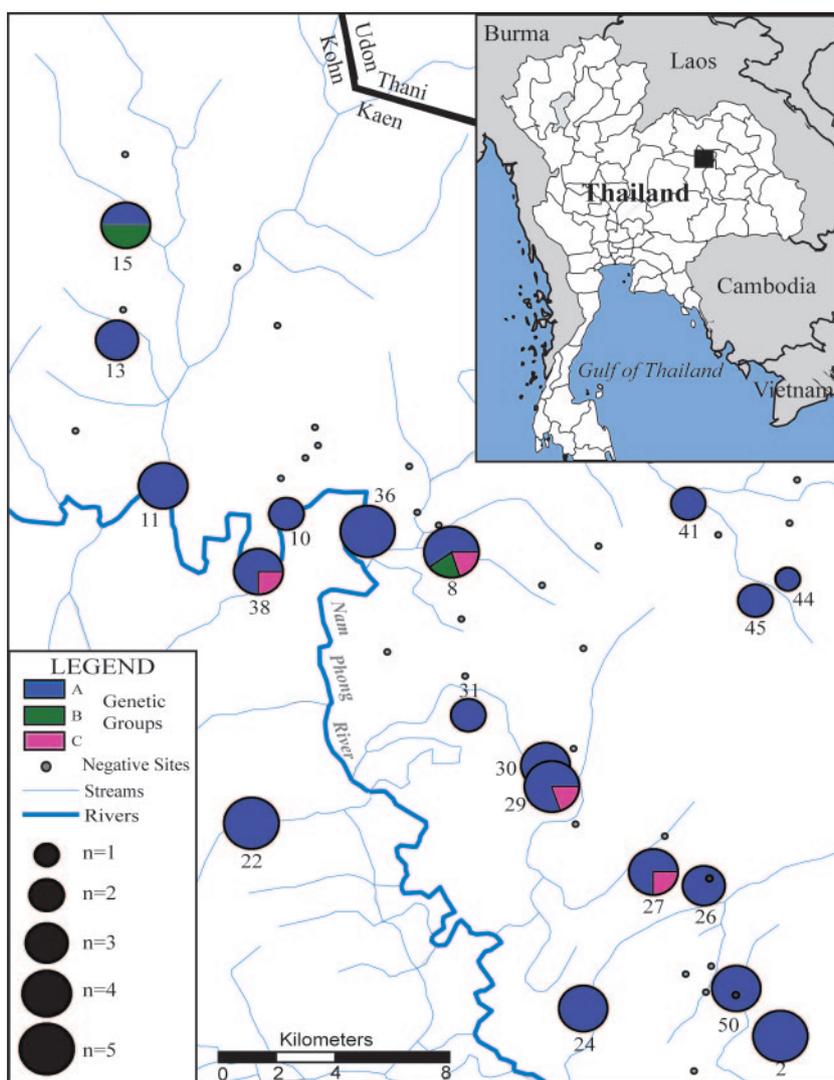

FIG. 2. Map of regional study area within Khon Kaen Province. The figure illustrates the local watershed, as well as the locations of the 50 sites from which soil samples were collected. Each site is associated with a local village within the province. The center of each circle represents the location of a positive sampling area. Sites that were not positive for *B. pseudomallei* are shown as a black circle. Pie charts represent the percentages of isolates among three genetic clades (A, B, and C) for the *B. pseudomallei* DNA samples. The size of the pie chart is indicative of the number of samples from that site, ranging from one to five.

The soil and associated environment represent the pathogen reservoir for *B. pseudomallei* and the primary source of melioidosis (3). Previous studies have demonstrated the link between environmental isolates and clinical isolates associated with disease in humans and animals (12). For example, a study in the nearby Thai province of Ubon Ratchathani found that clinical and environmental samples shared the same MLST STs, although genotype frequencies differed between the two categories (17). The most prevalent genotype in both categories was ST70 (17), which is associated with our MLVA genetic clades A and B in this study. In contrast, the ST associated with MLVA clade C is novel, although it is closely related to ST173 which is also linked with a human case of melioidosis. These results reinforce the perceived association between soil isolates and melioidosis infection in northeast Thailand.

There was no apparent spatial genetic differentiation, with members of all three clades distributed across the sampling area (Fig. 2). Genotypes within clade A were found at all sites, and the dominant genotype from this clade occurred in 17 of the 19 sites. Clade B genotypes were found at only two sites, and clade C genotypes were observed at four sites (Fig. 2). However, the Mantel test found no correlation between genetic distance and geographic distance for the isolates ($R^2 = 0.007$). Additionally there was no association between soil depth and genotype ($\chi^2 = 7.43$, df = 4, $P = 0.11$).

B. pseudomallei has been found to be both highly diverse even across very short geographic distances (9) and persistent in the environment for long periods of time (3). As such, genetic diversity at many geographic and temporal scales can be quite large (4, 7, 9, 16). In contrast, *B. pseudomallei* soil isolates collected for this study show lower levels of genetic diversity. This implies that there may have been a significant genetic bottleneck and subsequent population expansion in this region. The spatial homogeneity of genotypes supports this hypothesis of rapid dispersal and colonization, and given the sampling locations, the dispersal mechanism may likely be the flooding of the Nam Phong River during the rainy season.

The results of this study indicate that the overall diversity of *B. pseudomallei* in northeast Thailand may be a result of the cumulative effects of multiple locally differentiated subpopulations. However, further studies are needed to determine the population dynamics of *B. pseudomallei*, both over time and a wide geographic area. These studies, in combination with high-resolution subtyping methods, such as MLVA, will facilitate our understanding of the pathogen reservoir structure and how it contributes to melioidosis incidence.

REFERENCES

1. **Ashdown, L. R., and S. G. Clarke.** 1992. Evaluation of culture techniques for isolation of *Pseudomonas pseudomallei* from soil. Appl. Environ. Microbiol. **58:**4011–4015.

2. **Chaowagul, W., N. J. White, D. A. Dance, Y. Wattanagoon, P. Naigowit, T. M. Davis, S. Looareesuwan, and N. Pitakwatchara.** 1989. Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand. J. Infect. Dis. **159:**890–899.

3. **Cheng, A. C., and B. J. Currie.** 2005. Melioidosis: epidemiology, pathophysiology, and management. Clin. Microbiol. Rev. **18:**383–416.

4. **Cheng, A. C., D. Godoy, M. Mayo, D. Gal, B. G. Spratt, and B. J. Currie.** 2004. Isolates of *Burkholderia pseudomallei* from northern Australia are distinct by multilocus sequence typing, but strain types do not correlate with clinical presentation. J. Clin. Microbiol. **42:**5477–5483.

5. **da Silva Costa, J. L., and V. C. de Oliveira.** 2003. Enhancement of soil DNA extraction by the use of hand held mixer. Braz. J. Microbiol. **34:**311–312.

6. **Finkelstein, R. A., P. Atthasampunna, and M. Chulasamaya.** 2000. *Pseudomonas* (*Burkholderia*) *pseudomallei* in Thailand, 1964–1967: geographic distribution of the organism, attempts to identify cases of active infection, and presence of antibody in representative sera. Am. J. Trop. Med. Hyg. **62:**232–239.

7. **Godoy, D., G. Randle, A. J. Simpson, D. M. Aanensen, T. L. Pitt, R. Kinoshita, and B. G. Spratt.** 2003. Multilocus sequence typing and evolutionary relationships among the causative agents of melioidosis and glanders, *Burkholderia pseudomallei* and *Burkholderia mallei*. J. Clin. Microbiol. **41:**2068–2079.

8. **Peakall, R., and P. E. Smouse.** 2001. genalex6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes **8:**288–295.

9. **Pearson, T., M. U'Ren, J. J. M. Schupp, G. J. Allan, P. G. Foster, M. J. Mayo, D. Gal, J. L. Choy, R. L. Daugherty, S. Kachur, C. L. Friedman, B. Leadem, S. Georgia, H. Hornstra, A. J. Vogler, D. M. Wagner, P. Keim, and B. J. Currie.** 2007. VNTR analysis of selected outbreaks of *Burkholderia pseudomallei* in Australia. Infect. Genet. Evol. **7:**416–423.

10. **Rotz, L. D., A. S. Khan, S. R. Lillibridge, S. M. Ostroff, and J. M. Hughes.** 2002. Public health assessment of potential biological terrorism agents. Emerg. Infect. Dis. **8:**225–230.

11. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

12. **Sermswan, R. W., S. Wongratanacheewin, S. Trakulsomboon, and V. Thamlikitkul.** 2001. Ribotyping of *Burkholderia pseudomallei* from clinical and soil isolates in Thailand. Acta Trop. **80:**237–244.

13. **Suputtamongkol, Y., A. J. Hall, D. A. Dance, W. Chaowagul, A. Rajchanuvong, M. D. Smith, and N. J. White.** 1994. The epidemiology of melioidosis in Ubon Ratchatani, northeast Thailand. Int. J. Epidemiol. **23:**1082–1090.

14. **Swofford, D. L.** 1999. PAUP, phylogenetic analysis using parsimony (and other methods), version 4.0 Beta. Sinauer, Sunderland, MA.

15. **U'Ren, J. M., M. N. Van Ert, J. M. Schupp, W. R. Easterday, T. S. Simonson, R. T. Okinaka, T. Pearson, and P. Keim.** 2005. Use of a real-time PCR TaqMan assay for rapid identification and differentiation of *Burkholderia pseudomallei* and *Burkholderia mallei*. J. Clin. Microbiol. **43:**5771–5774.

16. **U'Ren, J. M., J. Schupp, T. Pearson, H. Hornstra, C. Clark, K. Smith, R. Leadem Daugherty, S. Rhoton, B. Leadem, S. Georgia, M. Cardon, L. Huynh, D. Deschazer, S. P. Harvey, R. Robison, B. Currie, and P. Keim.** 2007. Tandem repeat regions within the *Burkholderia pseudomallei* genome and their application for high resolution genotyping. BMC Microbiol. **7:**23.

17. **Vesaratchavest, M., S. Tumapa, N. P. J. Day, V. Wuthiekanun, W. Chierakul, M. T. G. Holden, N. J. White, B. J. Currie, B. G. Spratt, E. J. Feil, and S. J. Peacock.** 2006. Nonrandom distribution of *Burkholderia pseudomallei* clones in relation to geographical location and virulence. J. Clin. Microbiol. **44:**2553–2557.

18. **Vuddhakul, V., P. Tharavichitkul, N. Na-Ngam, S. Jitsurong, B. Kunthawa, P. Noimay, P. Noimay, A. Binla, and V. Thamlikitkul.** 1999. Epidemiology of *Burkholderia pseudomallei* in Thailand. Am. J. Trop. Med. Hyg. **60:**458–461.

19. **White, N. J.** 2003. Melioidosis. Lancet **361:**1715–1722.

20. **Wuthiekanun, V., D. A. Dance, Y. Wattanagoon, Y. Supputtamongkol, W. Chaowagul, and N. J. White.** 1990. The use of selective media for the isolation of *Pseudomonas pseudomallei* in clinical practice. J. Med. Microbiol. **33:**121–126.

21. **Yeates, C., M. R. Gillings, A. D. Davison, N. Altavilla, and D. A. Veal.** 1998. Methods for microbial DNA extraction from soil for PCR amplification. Biol. Proced. Online **1:**40–47.

22. **Young, C. C., R. L. Burghoff, L. G. Keim, V. Minak-Bernero, J. R. Lute, and S. M. Hinton.** 1993. Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. Appl. Environ. Microbiol. **59:**1972–1974.