Multilocus Sequence Typing of Clinical Isolates of *Burkholderia pseudomallei* Collected in Hainan, a Tropical Island of Southern China

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Abstract. *Burkholderia pseudomallei* is a gram-negative bacterium that causes melioidosis. In this study, we examined the sequence types (STs) of 60 clinical isolates from patients with melioidosis in Hainan, a tropical island in southern China. The 60 clinical isolates were resolved into 30 STs. Among the STs, ST562 was also reported in Australia, and ST90 was also reported in the United States, probably from a traveler who had previously visited Asia. In addition, six novel STs were found in this study, including ST1395, ST1396, ST1397, ST1398, ST1399, and ST1443, whereas the remaining STs were mostly shared with other southeast Asian regions. Phylogenetic analysis of 60 isolates conducted using the eBURST v3 software segregated the 30 STs into three groups and 18 singletons. Our study shows genetic diversity of 60 clinical isolates of *B. pseudomallei* in Hainan Island.

INTRODUCTION

Burkholderia pseudomallei is a gram-negative environmental saprophyte that causes melioidosis. This organism is commonly isolated from soil and water in endemic areas such as southeast Asia and northern tropical Australia.^{1–5} Melioidosis usually occurs in the rainy season, particularly after a rainstorm or typhoon.^{6–9} In endemic areas, the greatest risk of developing melioidosis arises from frequent exposure to contaminated soil, water, or environmental aerosols. *Burkholderia pseudomallei* infection may be acquired through inhalation, subcutaneous inoculation, or direct exposure of wounded skin to contaminated material.^{10–13} Clinical presentations of melioidosis are highly variable, and can manifest as asymptomatic infection, localized skin abscesses, acute or chronic pneumonia, genitourinary, bone, and joint infections, or severe systemic sepsis with or without multiple abscesses in internal organs. Septic shock cases have a greater than 90% mortality rate.^{14,15} Because of the nonspecific disease presentation, lack of an available vaccine, and fear of deliberate release coupled with a high mortality rate, *B. pseudomallei* was upgraded to a Tier 1 select agent by the Centers for Disease Control and Prevention in October 2012 (<http://www.selectagents.gov/>).

Hainan Island is the largest tropical island in southern China. Situated in southeast Asia, it is close to endemic regions such as Thailand, Vietnam, Laos, and Hong Kong. Hainan has a tropical monsoon maritime climate characterized by high temperature and humidity. The rainy season of Hainan typically occurs between May and October, when frequent rainstorms and typhoons occur (<http://www.weather.com.cn/hainan/qxjjj/03/341906.shtml>). These factors combined have allowed Hainan to become a region of melioidosis endemicity.¹⁶

Multilocus sequence typing (MLST) has been used to characterize the population structure and diversity of *B.*

pseudomallei; however, there is little information regarding this organism available from China (<http://pubmlst.org/bpseudomallei/>). Therefore, this study was conducted to analyze the sequence type (ST) diversity of clinical strains of *B. pseudomallei* from Hainan in China.

MATERIALS AND METHODS

Ethics statement. In this study, we anonymized the melioidosis patient data. All clinical isolates were collected during routine melioidosis laboratory diagnosis; therefore, patients did not provide written informed consent.

Clinical isolates. A total of 60 clinical strains of *B. pseudomallei* were acquired from culture-confirmed melioidosis patients admitted to Hainan General Hospital, Haikou, and Hainan Department of the General Hospital of the Chinese People's Liberation Army, Sanya. Information describing the 60 strains is shown in Table 1. Glycerol stocks of *B. pseudomallei* were stored at –80°C.

Bacterial diversity analysis. A total of 60 clinical isolates were typed by MLST as previously described.¹⁷ The number of alleles on seven housekeeping genes was determined by comparing the sequences to those at the *B. pseudomallei* MLST website (<http://pubmlst.org/bpseudomallei/>). The seven numbers were composed of the allelic profile of each strain, and each distinct allelic profile was assigned as a sequence type. New alleles and STs were submitted to the *B. pseudomallei* MLST database curator.

Phylogenetic analysis. The relatedness among 30 STs was displayed as a dendrogram by eBURST v3 (Ed Feil, University of Bath, Bath, United Kingdom) with six shared alleles defined as a group.¹⁸ We downloaded the entire STs and allelic profiles of *B. pseudomallei* on the MLST website as a reference file, then compared the data with those for the 30 STs of this study as a query file by eBURST v3. The novel STs in our study are highlighted green, and the existing STs are highlighted magenta.

RESULTS AND DISCUSSION

Information describing the 60 clinical isolates. The 60 strains included in this study were isolated from 60 melioidosis

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TABLE 1
Clinical information and STs of 60 clinical isolates of *Burkholderia pseudomallei* in this study

Strain	Sample type	Location	Occurrence	Main clinical presentation	Outcome	ST
HPPH 23	Blood	Haikou	November 2011	Septicemia	S	46
HPPH 32	Bone marrow	Baoting	July 2012	Septicemia, pneumonia	D	46
HPPH 44	Blood, secretion	Ledong	April 2013	Septicemia, pneumonia, abscess	D	46
HPPH 45	Blood, secretion	Ledong	May 2013	Abscess	S	46
HPPH 65	Blood	Dongfang	August 2014	Septicemia, pneumonia, abscess	D	46
HPPH 83	Sanies	Haikou	January 2004	Abscess	S	46
HPPH 93	Sanies	Changjiang	November 2013	Septicemia, pneumonia, abscess	D	46
HPPH 50	Blood	Wanning	September 2013	Septicemia, pneumonia	D	50
HPPH 55	Blood	Haikou	February 2014	Septicemia, pneumonia	S	50
HPPH 57	Blood, sanies	Wanning	April 2014	Septicemia, pneumonia, abscess	D	50
HPPH 72	Blood	Dingan	October 2014	Septicemia, pneumonia	S	50
HPPH 76	Sputum	Wanning	December 2014	Pneumonia	S	50
HPPH 40	Blood, sanies	Dongfang	December 2012	Septicemia, abscess	D	55
HPPH 47	Sputum	Dongfang	May 2013	Pneumonia	S	55
HPPH 51	Blood	Dongfang	September 2013	Septicemia, pneumonia, abscess	D	55
HPPH 61	Blood	Dongfang	July 2014	Septicemia, pneumonia	D	55
HPPH 92	Sanies	Dongfang	U	Abscess	S	55
101699	Tissue	Ledong	April 2013	Abscess	S	55
o91238	Blood	Sanya	October 2012	U	D	55
111506	Blood	Sanya	December 2014	U	S	55
HPPH 53	Blood, tissue	Wanning	October 2013	Septicemia, pneumonia	D	58
HPPH 80	Blood	Wenchang	October 2005	Septicemia	S	58
HPPH 81	Blood, sputum	Wanning	October 2005	Septicemia, pneumonia, abscess	D	58
141207009	Blood, sputum	Ledong	August 2013	Septicemia, pneumonia	D	58
141225021	Blood	Sanya	U	Septicemia, pneumonia	U	58
o8005	Blood	Sanya	September 2013	U	S	58
HPPH 42	Blood	Ledong	January 2013	Septicemia, pneumonia, abscess	D	70
HPPH 66	Blood	Haikou	August 2014	Septicemia, pneumonia, abscess	D	70
HPPH 69	Blood	Changjiang	September 2014	Septicemia, pneumonia	S	90
HPPH 86	Sanies	Dongfang	U	U	S	165
o42901	Sputum	Ledong	U	Pneumonia	U	205
HPPH 63	Blood	Haikou	August 2014	Septicemia, pneumonia, abscess	D	271
HPPH 75	Sanies	Qionghai	October 2014	Pneumonia, abscess	S	354
HPPH 77	Blood	Dongfang	December 2013	Septicemia, pneumonia	S	366
HPPH 43	Blood	Haikou	March 2013	Septicemia, pneumonia	D	562
HPPH 48	Blood	Tunchang	June 2013	Septicemia, pneumonia, abscess	D	562
HPPH 58	Blood	Dingan	July 2014	Septicemia, pneumonia	S	658
HPPH 62	Sanies	Wenchang	July 2014	Abscess	S	658
HPPH 54	Tissue	Dongfang	December 2013	Abscess	S	677
HPPH 68	Sanies	Lingshui	September 2014	Abscess	S	677
112011	Sputum	Sanya	U	U	S	677
o83020	Blood	Sanya	December 2014	Septicemia, pneumonia	D	1091
HPPH 24	Sanies	Ledong	February 2012	Septicemia, pneumonia, abscess	S	1092
HPPH 21	Blood	Danzhou	October 2011	Septicemia	S	1093
HPPH 1	Blood, sputum	Lingao	June 2010	Septicemia, pneumonia	D	1094
HPPH 4	Blood	Sanya	October 2010	Septicemia, pneumonia	S	1095
HPPH 82	Blood	Haikou	August 2003	Septicemia, abscess	D	1096
HPPH 56	Sanies	Changjiang	February 2014	Pneumonia	S	1098
HPPH 67	Sanies	Wenchang	September 2014	Pneumonia	S	1105
HPPH 70	Blood	Haikou	October 2014	Septicemia, pneumonia, abscess	D	1105
HPPH 85	Blood, sanies	Wanning	U	Septicemia, abscess	S	1107
HPPH 59	Blood	Wenchang	July 2014	Septicemia	S	1325
HPPH 60	Blood	Wenchang	July 2014	Septicemia, pneumonia	D	1325
HPPH 73	Blood	Wenchang	November 2014	Septicemia, pneumonia	D	1443
HPPH 74	Sputum	Chengmai	November 2014	Pneumonia	S	1443
HPPH 49	Sputum	Ledong	July 2013	Pneumonia	S	1395
HPPH 52	Blood	Dingan	August 2013	Pneumonia	S	1396
HPPH 64	Blood	Qionghai	August 2014	Septicemia, pneumonia	D	1397
HPPH 71	Blood	Wenchang	October 2014	Septicemia, pneumonia	D	1398
HN008_J	Blood	Sanya	U	U	S	1399

D = died; S = survived; STs = sequence types; U = unclear. In heavily shaded rows, STs was only found in Hainan. ST1395–1399 and ST1443 were the novel STs found in this study. ST562 was reported in both China and Australia; ST90 was also reported in the United States, probably from a traveler who had previously visited Asia.

patients in 15 different regions of Hainan excluding Basha, Qiongzong, and Wuzhishan. All regions except Dingan, Tunchang, and Baoting were located along the coast (Figure 1). Because the population density of coastal areas was higher than that of inland areas, it remains unknown whether coastal areas are more conducive to *B. pseudomallei* growth and persistence

than inland regions. Environmental samples of both coastal and inland areas across Hainan are needed to better understand the true ecological distribution of *B. pseudomallei* on this island. In Hainan, the presentations of melioidosis mainly included pneumonia, abscess formation, and septicemia. Overall, 31 of 35 patients with septicemia died (Table 1).

FIGURE 1. Map of Hainan Island and distribution of the 30 sequence types (STs). Map obtained from <http://www.hinews.cn/news/system/2009/11/10/010605499.shtml> showing the major regions in the island. The STs of 60 clinical isolates were labeled in each region in which melioidosis patients come from. The number of the strains is shown in the brackets.

STs of 60 clinical isolates. The 60 clinical isolates belonged to 30 distinct STs, six of which had not been previously documented (Table 2). Among the six new STs, the *gmhD* locus of ST1443 was 99% similar to the allele-2 sequence, whereas the other new STs were generated by reassortment of existing alleles. These findings indicated that there was low genetic diversity within alleles, but alleles recombined frequently to form new STs. Among the 60 isolates, the loci of *ace*, *gltB*, *lepA*, *lipA*, and *ndh* had only three types of alleles, most of which were allele-1, but the loci of *gmhD* and *narK* had more than six alleles.

The remaining 24 STs were reported from other southeast Asian regions except ST562, ST90, and 10 STs that were only reported in China (Table 1). ST562 was shared with Australia and ST90 with the United States. It was previously reported that isolates of *B. pseudomallei* from Australia and

Asia were distinct by MLST.¹⁹ Even isolates from the different regions of Australia were distinct.^{20,21} Because *B. pseudomallei* had not naturally been found in the environment in the United States, ST90 was probably from a traveler who had previously visited Asia. The ST562 may also have been caused by homoplasmy in the MLST loci,²² or imported strains because Hainan is known for an international tourism. In view of the high level of genetic recombination, it may be a recombinant.²³

The ST distribution of 60 clinical isolates also differed (Figure 1). ST55 (eight strains), ST46 (seven strains), ST58 (six strains), and ST50 (five strains) were the most prevalent sequence types (Table 1). ST55 and ST46 were mainly found in southwest Hainan of Dongfang, Ledong, and Sanya, whereas ST58 was primarily distributed in southern Hainan and ST50 was primarily in northeastern Hainan. However,

Alleles types of six novel sequence types (STs)		Locus					
		<i>ace</i>	<i>gltB</i>	<i>gmhD</i>	<i>lepA</i>	<i>lipA</i>	<i>ndh</i>
ST	Strain						
1443	HPPH73, HPPH74	1	3	126 ^{new}	1	5	2
1395	HPPH 49	3	1	28	1	1	2
1396	HPPH 52	1	2	6	1	1	22
1397	HPPH 64	1	2	13	1	1	2
1398	HPPH 71	1	1	3	1	1	40
1399	HN008_J	1	4	3	1	1	4

^{new} = new sequence, 99% similarity of allele-2.

FIGURE 2. eBURST diagram of sequence types from isolates in this study. I = group 1; II = group 2; III = group 3.

FIGURE 3. eBURST diagram comparing *Burkholderia pseudomallei* isolates from this study to those from the entire world. The novel sequence types (STs) in this study are highlighted green, and the existing STs are highlighted magenta.

more clinical isolates and environmental samples are needed to confirm these findings.

The relatedness of 30 STs. The 30 STs were divided into three groups and 18 singletons by eBURST. The majority of STs in the present study were singletons. With the exception of group 2, they did not form a radial expansion pattern (Figure 2). The 30 STs were compared with all of the STs present in the MLST database using eBURST (Figure 3). The results demonstrated a high level of diversity, with the 30 STs spread throughout almost all branches of the eBURST diagram.

In conclusion, the 60 clinical isolates from Hainan had a high level of diversity. Allele recombination may be an important factor leading to these diversities, and migration of humans may have also influenced the distribution of *B. pseudomallei* clones throughout Hainan.

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