

## Multilocus sequence typing of 102 *Burkholderia pseudomallei* strains isolated from China

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### SUMMARY

The phylogenetic and epidemiological relationships of 102 *Burkholderia pseudomallei* clinical isolates from different geographical and population sources in China were investigated by multilocus sequence typing (MLST). The MLST data were analysed using the e-BURST algorithm, and an unweighted pair-group method with arithmetic mean dendrogram was constructed based on the pairwise differences in the allelic profiles of the strains. Forty-one sequence types (STs) were identified, of which eight were novel (ST1341, ST1345, ST1346, ST1347, ST1348, ST1349, ST1350, ST1351). No geographical-specific or host population-specific phylogenetic lineages were identified. ST46, ST50, ST55, ST58, ST70 and ST1095 predominated, but ~44% of isolates were assigned to 45 STs illustrating high genetic diversity in the strain collection. Additionally, the phylogenetic relationships of the dominant STs in China showed significant linkage with *B. pseudomallei* isolates from Thailand. Analysis of the *gmhD* allele suggests high genetic variation in *B. pseudomallei* in China.

**Key words:** *Burkholderia pseudomallei*, e-BURST, multilocus sequence typing (MLST).

### INTRODUCTION

*Burkholderia pseudomallei* is the causative agent of melioidosis, a severe, often fatal infection of animals and humans [1]. *B. pseudomallei* infections occur most frequently in Southeast Asia and Northern Australia, but other regions of endemicity are emerging as the disease becomes more widely recognized

[2–6]. Furthermore, *B. pseudomallei* has been designated a select agent (Tier 1) by the U.S. Centers for Disease Control and Prevention (CDC) since 2006 because of its potential as a bioweapon [2]. Clinical manifestations of melioidosis vary greatly from localized abscesses to severe pneumonia or life-threatening sepsis, with reported mortality rates up to 40% and relapse rates of ~20%, regardless of antibiotic treatment [7, 8].

China has long been an endemic focus of melioidosis particularly in Hainan Province [9–11] which covers an area of 33 210 km<sup>2</sup> and has a population of over

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Table 1. Primers used for *B. pseudomallei* MLST

Gene	Gene product	Direction	Oligonucleotide sequence (5'–3')
<i>ace</i>	Acetyl-CoA acetyltransferase	F	GAATCGCCTTCACCATGTC
		R	CCGCGCTTCTCAAACGATA
<i>gltB</i>	Glutamate synthase, large subunit	F	ACGCTCGCGATCGCGATGAA
		R	TTCAGCAGGAGCGTCTGCTG
<i>gmhD</i>	ADP-L-glycero-D-manno-heptose-6-epimerase	F	<u>GCAGTTCCTGTATGCGTA</u>
		R	<u>GAAGCACTGGTACTTGCC</u>
<i>lepA</i>	GTP-binding protein	F	CATATTCGCAATTTCTCGATC
		R	CACGAGCATCACGACGCCG
<i>lipA</i>	Lipoyl synthase	F	<u>TCCGATCAAGATCGTCCCGA</u>
		R	<u>AGGTATTGGCCGATCGTCAG</u>
<i>narK</i>	Nitrite extrusion protein	F	CTACTCGTGCGCTGGGAT
		R	GACGATGACGGCACCCAC
<i>ndh</i>	NADH dehydrogenase subunit ENADH-quinone oxidoreductase, F subunit	F	AGTCGCGACGTTCTACAC
		R	CGAGTTGCAGACGAGATAT

Underlined sequences indicate new primers.

nine million native people (Census data, 2014) in addition to a significant number of foreigners. Although national data on the regional distribution of melioidosis cases, is lacking, a recent literature review revealed that almost all reported cases in China have occurred in Hainan [12], hence the importance of this province for the study of *B. pseudomallei*.

Multilocus sequence typing (MLST) has been applied to the molecular epidemiological study of *B. pseudomallei* since 2003 [13]. The technique generates data that are easy-to-use, unambiguous, and readily comparable across laboratories. To date, about 1353 sequence types (STs) of *B. pseudomallei* have been identified worldwide, which reflects the high diversity within the species. However, the ST distribution across different countries as displayed in the MLST database, shows the dominance of ST36 and ST109 in Australia and ST70 and ST93 in Thailand, and furthermore, has revealed unsuspected epidemiological linkages of *B. pseudomallei* in remote endemic regions and unrelated geographical locations [14].

As no published study has yet applied MLST to *B. pseudomallei* isolates from Hainan we reasoned, given the prevalence of melioidosis in the province, that this approach would provide helpful insights and possibly a better understanding of the molecular epidemiology of this pathogen [12, 15]. In this study, we collected 102 *B. pseudomallei* strains with clear clinical documentation predominantly from Hainan over a 12-year period, and here present the clinical characteristics of patients and the results of MLST analysis of the strain collection.

## METHODS

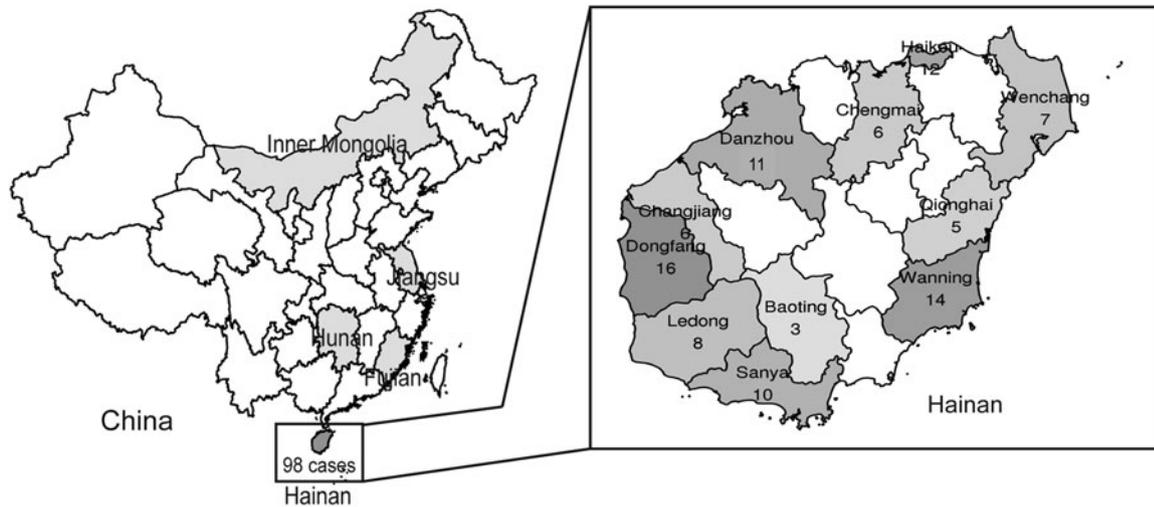
### Case collection and bacterial strains

One hundred and two strains of *B. pseudomallei* with a clear clinical background were collected between December 2002 and December 2014. Ninety-eight of these 102 strains were isolated in Hainan, and these were each from Fujian Province, the Inner Mongolia Autonomous Region, Hunan Province, and Jiangsu Province, but all cases were thought to have been acquired during travel to Hainan.

Most isolates were recovered from blood (63.7%), pus (23.5%), or sputum (11.8%) samples, and urine (4.9%) (Supplementary Table S1). Clinical specimens from suspected melioidosis cases on admission were cultured on Columbia blood agar at 37 °C for 2–3 days. DNA was extracted from sedimented cells from liquid culture of a single bacterial colony and subjected to polymerase chain reaction (PCR) to amplify the 16S rRNA gene [10] for species identity. Biochemical confirmation of *B. pseudomallei* was performed using the Vitek 2 Compact system (bioMérieux, USA).

### MLST

The PCR primers for amplification of seven house-keeping strains [13] are as listed in Table 1. The previously published *gmhD* and *lipA* primers were not always reliable in amplifying full-length amplicons, and so were replaced with new primers (underlined sequences in Table 1). For each locus, PCR was performed with denaturation at 95 °C for 5 min; 30 cycles



**Fig. 1.** Geographical distribution of *B. pseudomallei* cases from China. Map of the People's Republic of China (left) and Hainan Island (right). The number of cases in each district are shown.

of 95 °C for 30 s, annealing for 30 s (55 °C for *gmhD* and *lipA*, 60 °C for *ace*, *gltB*, *narK* and *ndh*, 62 °C for *lepA*) and 72 °C for 60 s; and a 7-min hold at 72 °C. The amplicons were sequenced using a 3700 DNA sequencer (Applied Biosystems, USA). The sequence data for each allele were trimmed to the correct length, searched online, and defined as relative allele numbers in the *B. pseudomallei* MLST database (<http://bpseudomallei.mlst.net/>). STs were assigned and strain numbers were deposited in the database.

#### Data analysis

Clinical characteristics and patients' demographic data were analysed in Excel (Microsoft Corp., USA). The phylogenetic relationships of all strains were generated using eBURST v. 3 ([eburst.mlst.net/v3/mlst\\_datasets/](http://eburst.mlst.net/v3/mlst_datasets/)) and diagrams of the study strains and all deposited strains of *B. pseudomallei* in the database were exported with JAVA v. 8.0 (<https://java.com/en/download/faq/java8.xml>). An unweighted pair-group method with arithmetic mean (UPGMA) dendrogram was constructed based on the pair-wise differences in the allelic profiles of our own MLST data and the reference STs (ST36 and ST109 representing strains from Australia, ST70 and ST93 from Thailand and ST246 from New Guinea). Trees inferred from allelic variations were produced using CLC Genomics Workbench v. 3.0 (Qiagen, USA) (based on UPGMA), to illustrate variation among some MLST loci.

#### Ethical standards

This study was approved by the Human Research Ethics Committee of the Third Military Medical

University, which is a member of the Chongqing City Ethics Committees of China. All clinical cases were anonymized without personal information and therefore, written informed consent was not required or obtained from the patients involved.

## RESULTS

#### Case locations and clinical characteristics

Ninety-eight of the 102 melioidosis cases were from the Hainan province and their distribution, according to 11 districts of Hainan, is shown in Figure 1. The four other cases were associated with previous travel in Hainan, and were accordingly considered to originate from the province. Prevalences ranged from 3 to 16 cases and five districts accounted for  $\geq 10$  cases. No case was recorded in the four inland cities of Hainan Island.

Cases ranged in age from 1 to 10 years (mean 48 years) and ~61% were defined as middle aged, i.e. aged >45 years. The majority (87.3%) were male and more than half were farmers or fishermen (data not shown). Sepsis (39.2%) and pneumonia (32.4%) were the major manifestations of melioidosis with few cases of abscesses (14.7%) and skin infections (13.7%). Twenty-two deaths were recorded and attributed primarily to sepsis (37.5%) and pneumonia (21.2%).

#### MLST analysis

All strains were resolved into 41 STs, eight of which were novel (ST1341 and ST1345–ST1351) (Supplementary Table S1). Eleven STs that were first

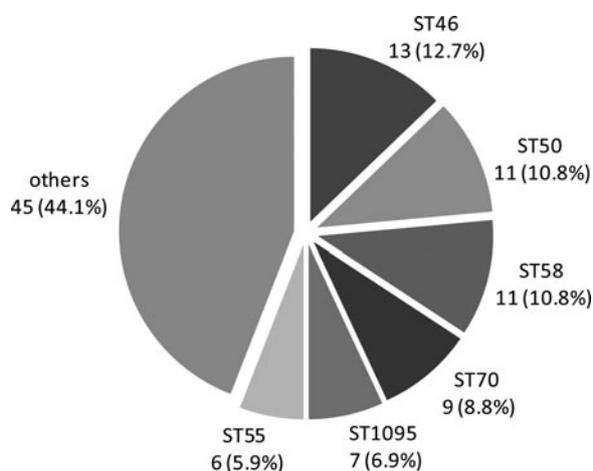


Fig. 2. MLST profiles of the 102 *B. pseudomallei* strains from China.

identified in China, i.e. ST1090 (1 case), ST1093 (1), ST1094 (1), ST1095 (4), ST1341 (2), ST1345 (1), ST1346 (1), ST1347 (1), ST1349 (1), ST1350 (1), and ST1351 (1), were associated with 12 of the 22 deaths (Supplementary Table S1).

The dominant STs occurring in  $\geq 5$  cases were ST46 (13 cases, 12.7%), ST50 (11, 10.8%), ST58 (11, 10.8%), ST55 (6, 5.9%), ST70 (9, 8.8%) and ST1095 (7, 6.9%); the remainder were associated with  $\leq 4$  cases (Fig. 2). Three STs (ST50, ST55, ST1095) were first identified in China (24 strains, 23.5%), followed by ST58 and ST70 (19.6%), first identified in Thailand, and ST46 (12.7%), first identified in Bangladesh.

Based on the pair-wise differences in the allelic profiles of the MLST data, several STs that were recently identified by Chen *et al.* [12] were included (shown as green circles in Fig. 3a), and other STs identified in the present study are displayed in Figure 3b. Based on the more comprehensive view provided by the *B. pseudomallei* MLST database, the STs of the study strains were too diverse to be grouped into a single clonal complex (CC) (Fig. 3b). Generally, ST46 and ST50 were grouped as a single CC, ST55 and ST58 in another CC, the new STs (ST1345, ST1346, ST1347, ST1350, ST1351) fell into two additional CCs. A UPGMA tree based on this study data and reference ST36 and ST109 (Australia), ST70 and ST93 (Thailand) and ST246 (New Guinea) showed significant linkage of isolates from China with those from Thailand, but only a weak relationship with strains from Australia as illustrated by ST1090 and ST109 (Australia) and between ST1349 (present study) and ST562, which was recently reported to be shared between strains from China and Australia [12]. ST1348,

ST246, and ST36 were clustered together, suggesting a possible correlation between *B. pseudomallei* from Australia and New Guinea strains, and these three STs were similar to those recently identified in China, including ST1094, ST1095, ST1106, and ST1341, which taken together might suggest a relationship between strains from Australia and China (Fig. 4).

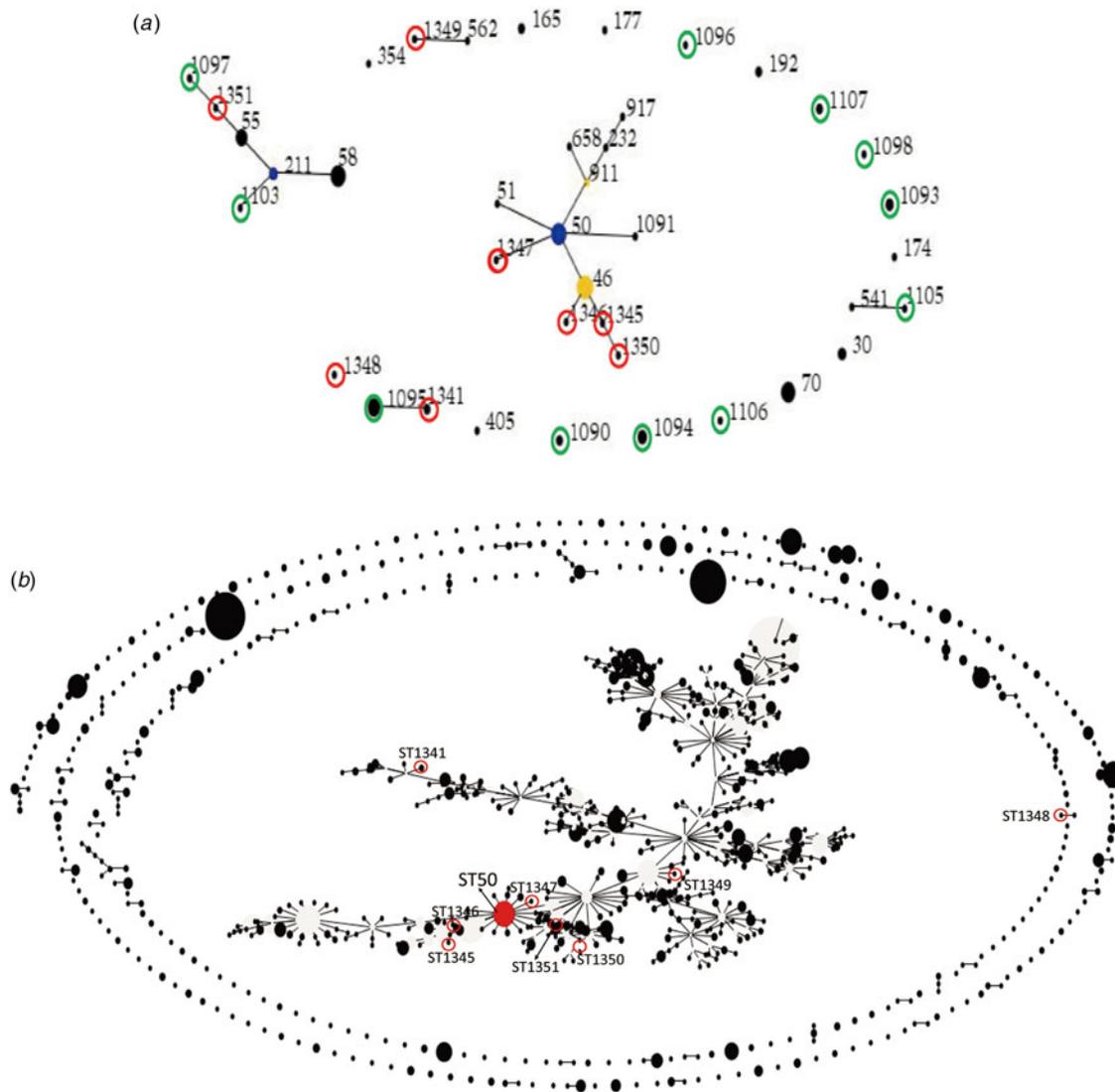
Some alleles were dominant in our collections, particularly *ace-3*, *gltB-1*, *gmhD-2*, *lepA-1*, *lipA-1*, *narK-4* and *ndh-1* (Table 2). Other alleles appeared to be rare, such as *ace-4* (specific to ST1093), *lepA-18* (ST1348), *lepA-4* (ST1090), *lipA-8* (ST1106), *gmhD-14* (ST1341 and ST174) and *ndh-11* (ST1348). Allele *gmhD* was the most variable with nine single locus variants while *ace* and *lipA* were the least variable with three single locus variants (Table 2). ST1348 was suspected to be an imported type as its allelic profile (1-2-13-18-1-22-11) was similar to ST246 (1-2-22-18-1-22-11), a clinical strain isolated from New Guinea in 1992, except for the *gmhD* allele. Other STs which differed only by the *gmhD* allele were ST1349, ST562, ST1341 and ST1095. It is notable that *gmhD* (28-4-6-14-13) occurred only in novel *B. pseudomallei* STs in China and a similar trend was not found for other alleles (Table 2).

## DISCUSSION

*B. pseudomallei* was once thought to be restricted to tropical regions, but is increasingly being recognized in other geographical locations [8]. Melioidosis is an emerging disease in Hainan being located within the tropical zone of melioidosis-endemic countries, such as Thailand, Vietnam, and Laos [10]. Indeed, most human melioidosis cases in China have been identified, treated and recorded in Hainan, and the increasing annual number of cases reported here underscores its prevalence in the province [9, 12]. It is possible that the number of cases in this study represents only a small proportion because of clinical unfamiliarity with the disease in Hainan [15].

Sepsis and pneumonia are the major manifestations of melioidosis in China which is consistent with other endemic regions [16]. Our rural patient population was predominantly middle-aged males and who often worked outdoors and possibly were more likely to have unhealthy lifestyles that predispose them to be immunocompromised, making them more susceptible to melioidosis.

MLST is an effective technique for molecular epidemiological studies of several bacterial species based on sequence variation of selected housekeeping

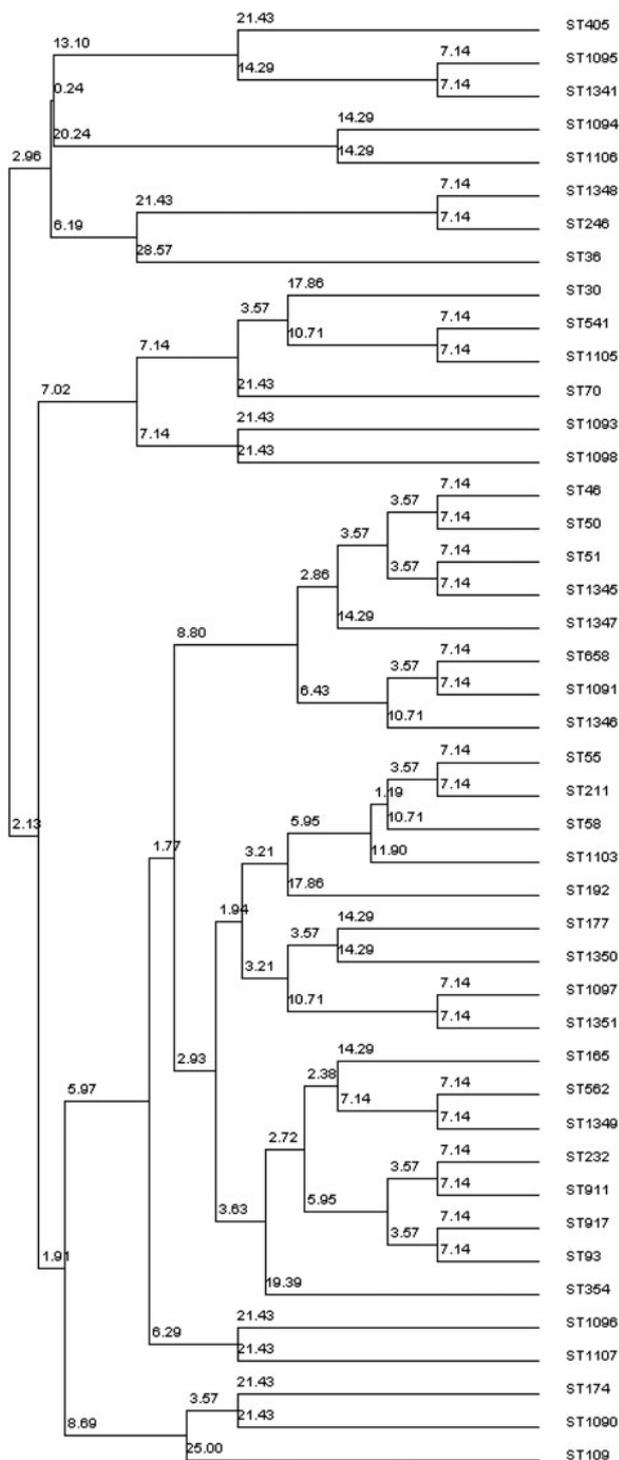


**Fig. 3.** eBURST of *B. pseudomallei* strains. (a) MLST data from present study. The primary founder (yellow), ST46, is positioned centrally in the cluster with subgroup founders (blue). Strain groups are connected by dark lines and novel STs from this study are represented by red circles; green circles represent STs previously found in China and submitted to the MLST database by the Chinese Centre of Disease Control. Dot diameter reflects the number of cases. (b) eBURST of all *B. pseudomallei* STs, including this study, based on the MLST database. Novel STs are highlighted by red circles with founder ST50 depicted as a red solid dot. Dot diameter reflects the number of cases.

gene loci [17]. The *B. pseudomallei* strains examined in this study proved to be phylogenetically diverse with 41 different STs, eight of which were novel, identified in the collection. No direct correlation was evident between STs and geographical location, isolation source, initial symptom or clinical outcome of the patients. Nevertheless, three novel STs (ST1346, ST1350, ST1351) were isolated from a single location (Dongfang City). However, the majority of deaths were due to strains of STs that were first identified in China, including ST50 which accounted for four deaths compared to single cases of several other STs

(Supplementary Table S1). All patients who died had *B. pseudomallei*-positive blood samples at admission or later during treatment (Supplementary Table S1), which reinforces the high mortality associated with bloodstream infection due to the organism.

The prevailing view of workers in the field is that strain lineages of *B. pseudomallei* possibly originated from Australia and subsequently spread to Southeast Asia and other regions [2, 14]. Our results only suggest a link between *B. pseudomallei* from Thailand and China (Figs 3 and 4) but apart from ST562, no



**Fig. 4.** UPGMA tree based on the matrix of pairwise differences in the MLST allelic profiles of the 102 *B. pseudomallei* strains. Reference STs (ST36 and ST109 from Australia, ST70 and ST93 from Thailand, and ST246 from New Guinea) are included in the tree.

other ST was shared between strains from China and Australia. This may be a true reflection of the great distance between the two countries but human

**Table 2.** Prevalence of allele numbers in the studied *B. pseudomallei* strains

Locus	Allele number and prevalence (%)
<i>ace</i>	3 (63.73), 1 (33.33), 4 (2.94)
<i>gltB</i>	1 (57.84), 4 (24.51), 2 (9.8), 12 (7.84)
<i>gmhD</i>	2 (31.37), 3 (25.49), 11 (11.76), 5 (9.8), 28 (7.84), 4 (3.92), 6 (3.92), 14 (2.94), 13 (2.94)
<i>lepA</i>	1 (55.88), 3 (30.39), 2 (10.78), 4 (1.96), 18 (0.98)
<i>lipA</i>	1 (79.41), 5 (19.61), 8 (0.98)
<i>narK</i>	4 (54.9), 2 (9.8), 3 (18.6), 1 (5.88), 22 (4.9), 9 (3.92), 29 (1.96)
<i>ndh</i>	1 (45.1), 3 (45.1), 6 (8.82), 11 (0.98)

movement cannot be discounted and raises new questions about the epidemiology and control of melioidosis [12]. However, similar or identical MLST profiles do not always signify shared identity of strains and it is possible that further deep sequencing of other gene loci might have revealed further differences between strains of the same ST [18].

Cases of imported melioidosis through travel of individuals from endemic areas have been documented in the literature [19–22] and during the 2008 Beijing Olympic Games, a case of Australian-imported melioidosis was reported by Xu *et al.* [10]. In the present study, ST1348 was associated with a probable imported case from New Guinea, and further investigation of this patient’s travel history is necessary. Hainan was labelled as an international tourist island 5 years ago, and attracts millions of overseas tourists every year, which presents a new challenge to public health authorities and the potential spread of *B. pseudomallei* to other places. This risk is illustrated by our identification of four cases who were diagnosed in regions outside of Hainan, but who had previously travelled in Hainan. It is possible that in the near future, melioidosis cases acquired in Hainan may be reported in other countries.

*gmhD* was the most variable allele of the seven house-keeping genes, and was the distinguishing feature between ST562 and ST1349, and between ST1348 and ST246. We speculate that variation in this allele may be responsible for the high genetic diversity of STs observed in this study. Additionally, as five allelic types of *gmhD* (28, 4, 6, 14, 13) were identified only in novel STs, this might suggest a role of *gmhD* mutation in the evolution of *B. pseudomallei* strains in China.

In conclusion, the wide distribution of *B. pseudomallei* in endemic areas and its high transmissibility, makes it likely that cases of melioidosis may arise in

more districts in China in the future. There has been a lack of MLST studies of the epidemiology of *B. pseudomallei* in Asia but our study has illustrated the emergence of novel STs of strains in China and further investigations are required to give a better understanding of the molecular epidemiology of this relatively rare infection in this country and inform health control measures.

## SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit <http://dx.doi.org/10.1017/S0950268815003052>.

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## DECLARATION OF INTEREST

None.

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