Multilocus sequencing-based evolutionary analysis of 52 strains of Burkholderia pseudomallei in Hainan, China

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Abstract

Previously, we reported a phylogenetic study of 98 Burkholderia pseudomallei clinical isolates from Hainan, China. Here, we update the B. pseudomallei strain library with 52 strains from newly identified cases dating from 2014 to 2017, analysed by multilocus sequence typing. Twenty-two sequence types (STs) were identified from the 52 cases, illustrating high genetic diversity; five of them (ST1480, ST1481, ST1482, ST1483 and ST1484) were novel. ST46, ST50 and ST58 predominated (34.6%) as was the case in the previous study (35.7%). An e-BURST map of the ST profiles of the two collections of isolates showed their genetic foundation to be largely unchanged. Neighbour-joining tree analysis was suggestive of a close phylogenetic relationship between the novel STs from this series and those first reported from Hainan (ST1105, ST1099, ST55 and ST1095). Moreover, the two novel STs (1481 and 1483) showed close similarity to ST58 which originated in Thailand indicating a close relationship between B. pseudomallei strains from both countries. The previously described allele profiles gmhD36 and lppA68 were found for the first time in our strain collections. Our study emphasises the importance of monitoring the epidemiological status and evolutionary trends of B. pseudomallei in China.

Burkholderia pseudomallei is a select agent (Tier 1) as classified by the US Centers for Disease Control and Prevention (CDC), which can infect both animals and humans and causes the disease melioidosis [1]. Melioidosis was generally thought to be endemic in Southeast Asia and Northern Australia but is now known to be distributed on a much wider scale [2–5]. Clinical manifestations of melioidosis vary greatly, from pneumonia to sepsis, with a high mortality rate of 40%, and a clinical relapse rate up to 20% [6–8]. Hainan is the main melioidosis-endemic area of China and covers an area of 33 210 km² with a population of over 9 million residents [8–10].

Multilocus sequence typing (MLST) has been used for the molecular epidemiological study of B. pseudomallei since 2003, and MLST data have proved to be easy-to-use, unambiguous and readily comparable across laboratories [11]. To date, approximately 1453 sequence types (STs) of B. pseudomallei have been identified worldwide, and the number of new cases identified by MLST increases each year. We have adopted this methodology as a standard analysis strategy for all B. pseudomallei strains isolated from cases of melioidosis in Hainan and previously published a phylogenetic and epidemiological study on 98 such isolates (2002–2014) [11]. Here, we report further phylogenetic analysis of 52 strains from cases in the province typed by MLST in the last 3 years to examine their genetic stability over time.

Fifty-two B. pseudomallei strains were isolated from melioidosis cases between January 2014 and August 2017 in Hainan Island; two were first isolated from non-coastal cities (Baisha and Qiongzhou). Most isolates were recovered from blood (63.5%) and pus (34.6%), others were from sputum (3.8%) and urine samples (3.8%) (Table 1). Clinical samples were cultured on Columbia blood agar incubated at 37°C for 2–3 days. B. pseudomallei identification was confirmed by the Vitek 2 Compact system (BioMerieux, Missouri, USA), and 16S rRNA PCR as previously described [11].

For MLST, PCR amplification of seven housekeeping genes was performed as previously published [11]. The sequence data for each allele were trimmed to a determined length and defined as relative allele numbers according to the B. pseudomallei MLST database (https://pubmlst.org/bpseudomallei). STs were assigned and all strain numbers were deposited in the database.

Clinical characteristics and patient demographic data were analysed in Microsoft Excel 2016. The phylogenetic relationships of all strains were generated using e-BURST v3 and
| Strain  | Year | ST<sup>a</sup> | ace | gltB | gltD | lepA | lipA | narK | ndh | Region of China<sup>b</sup> | Source | Manifestation |
|---------|------|----------------|-----|-------|-------|------|------|------|----|----------------------------|---------|----------------|
| BPC102  | 2014 | 1104           | 3   | 1     | 36    | 3    | 5    | 2    | 3  | Haikou                    | Blood   | Pneumonia       |
| BPC103  | 2014 | 658            | 1   | 4     | 2     | 1    | 1    | 4    | 3  | Haikou                    | Blood   | Septicaemia      |
| BPC104  | 2014 | 58             | 3   | 1     | 5     | 1    | 1    | 4    | 1  | Sanya                      | Sputum  | Septicaemia      |
| BPC105  | 2014 | 1096           | 3   | 4     | 28    | 1    | 1    | 3    | 1  | Changjiang                 | Pus     | Pneumonia       |
| BPC106  | 2014 | 46             | 3   | 1     | 2     | 1    | 1    | 3    | 3  | Haikou                    | Blood   | Pneumonia       |
| BPC107  | 2014 | 58             | 3   | 1     | 5     | 1    | 1    | 4    | 1  | Danzhou                    | Urine   | Pneumonia       |
| BPC108  | 2014 | 1105           | 1   | 4     | 2     | 3    | 5    | 4    | 1  | Baisha                     | Pus     | Pneumonia       |
| BPC109  | 2014 | 177            | 1   | 1     | 4     | 3    | 1    | 3    | 1  | Changjiang                 | Blood   | Sepsis          |
| BPC110  | 2014 | 1094           | 1   | 2     | 28    | 3    | 1    | 9    | 3  | Danzhou                    | Blood   | Parotid infection |
| BPC111  | 2014 | 58             | 3   | 1     | 5     | 1    | 1    | 4    | 1  | Ledong                     | Blood   | Sepsis          |
| BPC112  | 2014 | 354            | 1   | 1     | 3     | 2    | 1    | 4    | 1  | Haikou                     | Blood   | Pneumonia       |
| BPC113  | 2014 | 1480           | 3   | 3     | 2     | 1    | 1    | 3    | 3  | Dongfang                   | Blood   | Sepsis          |
| BPC114  | 2015 | 1099           | 1   | 12    | 3     | 2    | 1    | 2    | 1  | Haikou                     | Blood   | Pneumonia       |
| BPC115  | 2015 | 1095           | 1   | 12    | 3     | 2    | 1    | 2    | 3  | Haikou                     | Pus     | Pneumonia       |
| BPC116  | 2015 | 1106           | 1   | 2     | 28    | 3    | 8    | 4    | 3  | Wenchang                   | Pus     | Sepsis          |
| BPC117  | 2015 | 50             | 3   | 1     | 2     | 1    | 1    | 4    | 3  | Dongfang                   | Pus     | Pneumonia       |
| BPC118  | 2015 | 58             | 3   | 1     | 5     | 1    | 1    | 4    | 1  | Sanya                      | Pus     | Pneumonia       |
| BPC119  | 2015 | 658            | 1   | 4     | 2     | 1    | 1    | 4    | 3  | Sanya                      | Blood   | Pneumonia       |
| BPC120  | 2015 | 1108           | 3   | 1     | 2     | 68   | 1    | 4    | 3  | Sanya                      | Blood   | Pneumonia       |
| BPC121  | 2015 | 58             | 3   | 1     | 5     | 1    | 1    | 4    | 1  | Sanya                      | Sputum  | Sepsis          |
| BPC122  | 2015 | 70             | 3   | 4     | 11    | 3    | 5    | 4    | 6  | Sanya                      | Blood   | Soft tissue infection |
| BPC123  | 2015 | 50             | 3   | 1     | 2     | 1    | 1    | 4    | 3  | Sanya                      | Pus     | Soft tissue infection |
| BPC124  | 2015 | 562            | 1   | 1     | 4     | 1    | 1    | 29   | 1  | Sanya                      | Pus     | Pneumonia       |
| BPC126  | 2015 | 1481           | 3   | 1     | 5     | 1    | 5    | 4    | 1  | Qionghai                   | Pus     | Pneumonia       |
| BPC127  | 2015 | 1482           | 3   | 4     | 2     | 3    | 5    | 4    | 6  | Sanya                      | Blood   | Pneumonia       |
| BPC128  | 2015 | 1483           | 1   | 1     | 11    | 1    | 5    | 4    | 1  | Qionghai                   | Blood   | Pneumonia       |
| BPC129  | 2015 | 50             | 3   | 1     | 2     | 1    | 1    | 4    | 3  | Sanya                      | Blood   | Pneumonia       |
| BPC130  | 2015 | 50             | 3   | 1     | 2     | 1    | 1    | 4    | 3  | Sanya                      | Blood   | Sepsis          |
| BPC131  | 2015 | 50             | 3   | 1     | 2     | 1    | 1    | 4    | 3  | Qionghai                   | Blood   | Pneumonia       |
| BPC132  | 2015 | 366            | 3   | 1     | 2     | 3    | 8    | 4    | 3  | Sanya                      | Pus     | Pneumonia       |
| BPC133  | 2015 | 306            | 1   | 2     | 3     | 1    | 1    | 2    | 1  | Sanya                      | Pus     | Pneumonia       |
| BPC134  | 2015 | 1091           | 3   | 4     | 2     | 1    | 1    | 4    | 3  | Sanya                      | Blood   | Pneumonia       |
| BPC135  | 2015 | 366            | 3   | 1     | 2     | 3    | 8    | 4    | 3  | Sanya                      | Blood   | Pneumonia       |
| BPC136  | 2015 | 930            | 1   | 1     | 3     | 2    | 5    | 1    | 1  | Sanya                      | Blood   | Pneumonia       |
| BPC137  | 2015 | 46             | 3   | 1     | 2     | 1    | 1    | 3    | 3  | Sanya                      | Blood   | Pneumonia       |
| BPC138  | 2015 | 46             | 3   | 1     | 2     | 1    | 1    | 3    | 3  | Sanya                      | Blood   | Pneumonia       |
| BPC139  | 2015 | 46             | 3   | 1     | 2     | 1    | 1    | 3    | 3  | Sanya                      | Blood   | Pneumonia       |
| BPC140  | 2015 | 46             | 3   | 1     | 2     | 1    | 1    | 3    | 3  | Sanya                      | Blood   | Pneumonia       |
| BPC141  | 2015 | 46             | 3   | 1     | 2     | 1    | 1    | 3    | 3  | Sanya                      | Blood   | Pulmonary abscess |
| BPC142  | 2015 | 46             | 3   | 1     | 2     | 1    | 1    | 3    | 3  | Sanya                      | Blood   | Pneumonia       |
| BPC143  | 2015 | 1109           | 1   | 2     | 2     | 1    | 1    | 4    | 1  | Qionghai                   | Blood   | Pneumonia       |
| BPC144  | 2015 | 658            | 1   | 4     | 2     | 1    | 1    | 4    | 3  | Sanya                      | Blood   | Pneumonia       |
| BPC145  | 2015 | 658            | 1   | 4     | 2     | 1    | 1    | 4    | 3  | Sanya                      | Blood   | Pneumonia       |

(Continued)
compared against all strains in the database with JAVA 8.0. The
STs of 98 strains previously described from Hainan [11] were
also analysed to show the relationship and mutational trend
between the two groups of strains. The interactive tree of life
(ITOL) v3 ([https://pubmlst.org/](https://pubmlst.org/)) was used
to display phylogenetic relationships. This tool generates neighbour-joining trees from concatenated nucleo-
tide sequences based on the pair-wise differences in the allelic
profiles of strains [12].

The distribution of melioidosis cases across the 17 city areas
of Hainan Island between the two surveys 2002–2014 (black)
and 2014–2017 (red) is shown in Supplementary Fig. S1. The
ages among the 52 more recent cases ranged from 1 to 79 years
(median – 47 years); the majority were male (76.9%) and farmers
(71.2%). Pneumonia (71.2%) and sepsis (13.5%) remained the
major manifestations of melioidosis, with some cases presenting
with abscesses (5.8%) and soft tissue infections (7.7%). Seven
deaths occurred following pneumonia or sepsis, and old age
(⩾ 58 years); two of the deaths were associated with novel STs
(Table 1).

All 52 strains were resolved into 22 STs, five of which (ST1480,
ST1481, ST1482, ST1483 and ST1484) were novel (Table 1). The
dominant STs occurring in ⩾ 4 cases were ST50 (seven cases;
13.5%), ST58 (six; 11.5%), ST46 (five; 9.6%) and ST658 (four;
7.7%); these four STs accounted for 42.3% of all cases and the
remainder were associated with ⩽3 cases. This distribution reflects
that found in the first survey where the dominant STs were ST46
(13 cases; 13.2%), ST50 (11; 11.2%) and ST58 (11; 11.2%),
accounting for 35.7% in all 98 strains of B. pseudomallei.

The pair-wise differences in the allelic profiles of the STs iden-
tified in both strain collections are shown in Table 1. Half of the cases were clustered into a single dominant CC,
with ST50 as founder, and ST46 as sub-founder. Likewise three
of the novel STs in the present study (ST1480, ST1481 and
ST1484) and five novel STs from the first study (ST1345,
ST1346, ST1347, ST1350 and ST1351) were grouped in the
same CC (Fig. 1b). Furthermore, some novel STs originated
from the same founders but generated different gene types:
ST1484, ST1345 and ST1350 (founder: ST51), ST1480 and
ST1346 (founder: ST46), ST1481 and ST1351 (founder: ST211)
(Fig. 1b). Additionally, ST1482 and ST1483 were grouped in
different CCs; ST1482 showed close linkage to both ST70 and
ST541, both of which were first isolated from Thailand and
ST1483 had a relatively close relationship with ST1349, an ST
related to ST562, which was shared by Australian and Chinese
strains of B. pseudomallei [10].

The neighbour-joining tree shows the phylogenetic relation-
ship among the 22 STs identified in strains from this study
(Fig. 2). The five novel STs were genetically close to ST1105,
ST1099, ST55 and ST58, and ST1096 (all first isolated from
China, except for ST58, which was first isolated from Thailand).
The dominant allele profiles were mostly the same as previously
found, except for ndh-3; allele ndh-1 predominated in the first
strain collection. Some rare alleles were evident between the
studies, such as gmhD-36 (ST1196) and lepA-68 (ST1108) (Supplementary
Table S1).

It is clear that B. pseudomallei is no longer restricted to tropical
regions but is increasingly found in non-endemic areas [6].
Melioidosis is relatively uncommon in China but most reported
human cases in the country are from the tropical Hainan island
[8–10], and as in other countries, pneumonia remains the most
common presentation of the disease [7].

MLST has been repeatedly shown to be a simple and share-
able strategy for molecular epidemiological studies of B. pseudo-
mallei in various regions [2, 11]. Previously, we published an
MLST study of 98 B. pseudomallei strains from Hainan, and
here we have characterised 52 recently isolated strains and

| Strain | Year | ST* | allele at the following locus | Source | Manifestation |
|--------|------|-----|-----------------------------|--------|--------------|
| BPC163 | 2016 | 50  | 3 1 2 1 1 4 3              | Sanya  | Pus          |
| BPC164 | 2016 | 50  | 3 1 2 1 1 4 3              | Sanya  | Blood        |
| BPC165 | 2016 | 211 | 3 3 3 1 1 4 1              | Sanya  | Blood        |
| BPC166 | 2016 | 1446| 3 1 3 1 1 4 1              | Ledong | Pus          |
| BPC167 | 2016 | 58  | 3 1 3 1 1 4 1              | Sanya  | Pus          |
| BPC168 | 2016 | 1111| 3 3 4 1 1 4 1              | Baoting| Blood        |
| BPC169 | 2016 | 2017| 3 3 4 1 1 4 1              | Baoxing| Blood        |
| BPC170 | 2016 | 1091| 3 3 4 1 1 4 1              | Lingshui| Blood        |
| BPC171 | 2016 | 55  | 3 3 4 1 1 4 1              | Ledong | Pus          |
| BPC172 | 2016 | 46  | 3 3 4 1 1 4 1              | Lingshui| Pus          |
| BPC173 | 2016 | 48  | 3 3 4 1 1 4 1              | Lingshui| Pus          |
| BPC174 | 2016 | 1484| 3 3 4 1 1 4 1              | Lingshui| Blood        |

*The numbers in bold represent the novel STs, the death cases are labelled with underscore.

The shaded regions represent the regions which were identified as isolation locations in Hainan Island.

Table 1. (Continued.)
identified five novel STs. An e-BURST map of the 150 strains from both collections showed a clonal cluster with ST50 (first identified in China) as the founder genotype and that the genetic structure had remained largely unchanged over the 16-year period. Neighbour-joining tree analysis also suggests that recently emergent novel STs are closely related to some of the historical STs first isolated from Hainan Island. The linkage of two of the novel STs (1481 and 1483) to ST58, originating from Thailand, which has a close interplay with China [13]. Hainan attracts millions of tourists per year, which likely increases the risk of spreading infection among wider populations. A few melioidosis cases related to a history of travel in

Fig. 1. e-BURST analysis of the *B. pseudomallei* strains. (a) MLST data of 52 STs in this study. The primary founder (yellow), ST48, is positioned centrally in the cluster and subgroup founder (blue) is ST50. Strain groups are connected by dark lines and the five novel STs are highlighted by red circles. Dot diameter reflects the number of cases. (b) e-BURST of sequence types for combined 150 strains from both surveys. Novel STs highlighted by red circles and green circles, respectively. Dot diameter reflects the number of cases.
Hainan have been recorded by local hospitals in recent years (unpublished observations). In addition, the communications and commerce activities between the countries may promote the interaction of *B. pseudomallei* with different genetic backgrounds, and possibly give rise to the evolution of new gene types [13, 14]. No correlation was found between STs and geographic location, isolation source and clinical outcome of the patients but further genomic analysis may reveal hitherto unrecognised relationships [15].

In conclusion, this study has confirmed the relative genetic stability of *B. pseudomallei* strains in Hainan over a 15-year period. With the exception of the appearance of some novel STs, the clonal populations have remained mostly conserved but further monitoring of population structures and evolutionary trends through cooperative research with other endemic areas in the region is warranted.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S0950268818002741.

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**Conflict of interest.** None.

**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 anonymised without personal information.

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