Antibiotic susceptibility of clinical *Burkholderia pseudomallei* isolates in northeast Thailand during 2015-2018 and the genomic characterization of β-lactam-resistant isolates

Running Title: Antibiotic resistance in *B. pseudomallei*

Shirley Hii Yi Fen1, Sarunporn Tandhavanant1, Rungnapa Phunpang2, Peeraya Ekchariyawat1,3, Natnaree Saiprom1, Claire Chewapreecha2,4,5, Rathanin Seng1, Ekkachai Thiansukhon6, Chumpol Morakot7, Narongchai Sangsa8, Sunee Chayangsu9, Somchai Chuanonont10, Kittisak Tanwisaid10, Wirayut Silakun11, Noppol Buasi12, Seksan Chaisuksan13, Tanin Hompleum14, Ploenchai Chetchotisakd15, Nicholas P.J. Day2,16, Wasun Chantratita17, Ganjana Lertmemongkolchai18,19, T. Eoin West20, Narisara Chantratita1,2#

1Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
2Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
3Department of Microbiology, Faculty of Public Health, Mahidol University, Bangkok, Thailand
4Wellcome Sanger Institute, Hinxton, UK
Bioinformatics and Systems Biology Program, School of Bioresource and Technology, King Mongkut’s University of Technology Thonburi, Bangkok, Thailand

Department of Medicine, Udon Thani Hospital, Udon Thani, Thailand

Department of Medicine, Mukdahan Hospital, Mukdahan, Thailand

Department of Medicine, Roi Et Hospital, Roi Et, Thailand

Department of Medicine, Surin Hospital, Surin, Thailand

Department of Medicine, Nakhon Phanom Hospital, Nakhon Phanom, Thailand

Department of Medicine, Buriram Hospital, Buriram, Thailand

Department of Medicine, Sisaket Hospital, Sisaket, Thailand

Department of Medicine, Khon Kaen Hospital, Khon Kaen, Thailand

Department of Surgery, Khon Kaen Hospital, Khon Kaen, Thailand

Department of Medicine, Srinagarind Hospital, Faculty of Medicine and Research and Diagnostic Center for Emerging Infectious Diseases (RCEID), Khon Kaen University, Khon Kaen, Thailand

Center for Tropical Medicine and Global Health, University of Oxford, UK

Center for Medical Genomics, Faculty of Medicine, Ramathibodi Hospital, Bangkok, Thailand

Department of Clinical Immunology, Faculty of Associated Medical Science, Khon Kaen University, Khon Kaen, Thailand

The Centre for Research and Development of Medical Diagnostic Laboratories, Khon Kaen University, Khon Kaen, Thailand

Division of Pulmonary, Critical Care & Sleep Medicine, Harborview Medical Center,
Abstract

Melioidosis is an often fatal infection in tropical regions caused by an environmental bacterium, *Burkholderia pseudomallei*. Current recommended melioidosis treatment requires intravenous β-lactam antibiotics such as ceftazidime (CAZ), meropenem (MEM) or amoxicillin-clavulanic acid (AMC) and oral trimethoprim-sulfamethoxazole. Emerging antibiotic resistance could lead to therapy failure and high mortality. We performed a prospective multicentre study in northeast Thailand during 2015-2018 to evaluate antibiotic susceptibility and characterize β-lactam resistance in clinical *B. pseudomallei* isolates. Collection of 1,317 *B. pseudomallei* isolates from patients with primary and relapse infections were evaluated for susceptibility to CAZ, imipenem (IPM), MEM and AMC. β-lactam resistant isolates were confirmed by broth microdilution method and characterized by whole genome sequence analysis, *penA* expression and β-lactamase activity. The resistant phenotype was verified via *penA* mutagenesis. All primary isolates were IPM-susceptible but we observed two CAZ-resistant and one CAZ-intermediate resistant isolates, two MEM-less susceptible isolates, one AMC-resistant and two AMC-intermediate resistant isolates. One of 13 relapse isolates was resistant to both CAZ and AMC. Two isolates were MEM-less susceptible. Strains DR10212A (primary) and DR50054E (relapse) were multi-drug resistant. Genomic and mutagenesis analyses supplemented with gene
expression and β-lactamase analyses demonstrated that CAZ-resistant phenotype was caused by PenA variants: P167S (N=2) and penA amplification (N=1). Despite the high mortality rate in melioidosis, our study revealed that *B. pseudomallei* isolates had a low frequency of β-lactam resistance caused by penA alterations. Clinical data suggest that resistant variants may emerge in patients during antibiotic therapy and be associated with poor response to treatment.

**Keywords:** β-lactam resistance, penA, Melioidosis, Genome, *Burkholderia pseudomallei*, Ceftazidime, Thailand

**Introduction**

*Burkholderia pseudomallei* is the causative agent for melioidosis, an often fatal disease with predicted global burden of 165,000 cases per year and 89,000 deaths worldwide [1]. Melioidosis-endemic regions including Southeast Asia and northern Australia account for up to 40% [2] and 10% [3] case fatality rate, respectively. Transmission routes include percutaneous inoculation, inhalation or ingestion of contaminated soil and water [4]. The most common clinical presentations include pneumonia and bacteremia (40-60%) [5]. *B. pseudomallei* is categorized as Tier 1 Select Agent by the Centers of Disease Control and Prevention (CDC) [6]. To date, there is no commercially available vaccine for melioidosis.

*B. pseudomallei* is intrinsically resistant to many antibiotics including penicillin, ampicillin, first and second-generation cephalosporins [5]. Recommended melioidosis
treatment is biphasic therapy: an intensive phase of at least 10-14 days intravenous (IV) β-lactam antibiotics, ceftazidime (CAZ) or meropenem (MEM) followed by an eradication phase of 3-6 months of oral trimethoprim-sulfamethoxazole (SXT) to eliminate residual bacteria (https://www.cdc.gov/melioidosis/treatment/index.html). Amoxicillin-clavulanic acid (AMC), a combination of a β-lactam and a β-lactamase inhibitor, is used as alternative in both acute and eradication therapy [7]. SXT, which has excellent tissue penetration, is also added to intensive phase treatment for neurological-involvement and deep-seated abscess [7].

CAZ alone is the first line drug against melioidosis in Thailand due to its efficacy and lower cost compared to carbapenems. In Australia, MEM is preferred for severe melioidosis cases and is switched back to CAZ upon patients reaching stable conditions [5]. Prolonged acute-phase therapy and oral SXT are required because these regimens have been associated with lower risk for relapse [8-11]. Given the intrinsic antimicrobial resistance of the pathogen and few other therapeutic alternatives, emerging resistance against the limited drugs could be fatal. Primary CAZ resistance despite rare has been reported at 0.1-1.5% in Thailand [12-14] and 0.6-2.4% in Malaysia [15-17]. Acquired resistance has been observed in patients who received prolonged or multiple courses of CAZ [13,18-23]. The currently known mechanisms conferring CAZ resistance in *B. pseudomallei* have been described in some studies. For examples, alterations of membrane-bound class A β-lactamase, *penA* (BPSS0946; current NCBI locus tag BPS_RS23870) include the following mechanisms: (i) elevated *penA* expression due to promoter mutation (-78G>A) [23-25]; (ii) gene duplication and amplification (GDA) event in *penA* chromosomal region [21,26]; (iii) *penA* point
mutation at C69Y [18,21,23,27], P167S [20,22,27] and D240G [25]. Our previous study revealed several CAZ-resistant isolates in Thai patients caused by deletion of β-lactam target, penicillin-binding protein 3 (PBP3) (BPSS1219; current NCBI locus tag BPS_RS25365) [19]. Elevated expression of class D β-lactamase, oxa was also observed in laboratory-generated CAZ-induced resistant mutants [28-29]. In addition, an amino acid change in PenA involving S72F [22,25,27] and T147A [25] was reported to confer resistance towards AMC or both AMC and IPM, respectively. Alterations in B. pseudomallei encoded resistance nodulation and cell division (RND) multi-drug efflux pumps AmrAB-OprA, BpeAB-OprB, BpeEF-OprC have been implicated in antibiotic resistance. For instance, isolates harboring mutations affecting AmrR, BpeR, BpeT and BpeS increased the minimum inhibitory concentration (MIC) of MEM and SXT [21, 24, 30-32].

In this study, we aimed to evaluate the performance of β-lactam antibiotics in melioidosis treatment in vitro. Antibiotic susceptibility testing was conducted on 1,317 clinical B. pseudomallei isolates from 1,304 patients in nine hospitals across northeast Thailand during 2015 - 2018. We used whole genome sequencing to investigate genotype and search for putative mutations in β-lactam-resistant B. pseudomallei strains. Antibiotic treatment of these patients was investigated. Genomic and functional characterization of penA were evaluated via reverse-transcription real-time PCR (RT-PCR), β-lactamase activity and mutagenesis.

Results
Initial screening of antibiotic susceptibility in *B. pseudomallei* isolates

Initial screening of antibiotic susceptibility for 1,317 *B. pseudomallei* isolates was performed at nine hospitals (Table 1) and subsequent evaluation was performed at the Faculty of Tropical Medicine, Mahidol University (FTM) by methods as described in Figure 1. Six hospitals reported resistant and intermediate resistant isolates for CAZ, AMC, MEM and IPM: Hospital A (N=3), Hospital C (N=1), Hospital D (N=1), Hospital E (N=1), Hospital H (N=4) and Hospital I (N=8) (Table 2). In Hospital A, one isolate, DR10212A, was both CAZ-resistant (CAZ-R) and MEM-resistant (MEM-R) and two isolates were AMC-intermediate resistant (AMC-I). Hospital D reported one ceftazidime-intermediate resistant (CAZ-I) isolate. Hospital H reported two MEM-R isolates and FTM found one isolate of AMC-R and AMC-I, respectively. For Hospital I, upon checking on hospital laboratory reports, we observed that Vitek®2 Advanced Expert System (AES) interpreted one isolate as CAZ-R, MEM-R and IPM-resistant (IPM-R), one as MEM-intermediate resistant (MEM-I) and five isolates as IPM-R. FTM observed an additional one MEM-I isolate by disk diffusion testing (DD). We also found one isolate reported as CAZ-R at Hospital C and one isolate with both CAZ-R and AMC-R at Hospital E.

Validation of antibiotic susceptibility of *B. pseudomallei* isolates

Broth microdilution dilution (BMD) assessment was used to validate the antibiotic non-susceptibility and discrepant results of initial screenings between hospitals and FTM
for CAZ, MEM, IPM, AMC and SXT. Six isolates from Hospital I originally reported to be IPM-R were found at FTM to have MICs within the susceptible range (Table 2).

Three clinical isolates were confirmed as CAZ-R (strains DR10212A, DR30013A and DR50054E) with MIC of 128, 64 and 64 µg/ml respectively and one isolate (DR80110A) that was initially determined to be CAZ-S was re-interpreted as CAZ-I with an MIC of 16 µg/ml (Table 3). DR10212A, DR30013A and DR80110A were defined as primary isolates. We observed that the relapse isolate (DR50054E) had an increased MIC of CAZ of 64 µg/ml compared to the first episode (DR50054A) exhibiting an MIC of only 2 µg/ml.

The BMD results for MEM confirmed four isolates as MEM-less susceptible (MEM-LS) with MICs of 4 µg/ml (DR90049A, DR50054E and DR90031E) and 8 µg/ml (DR10212A) (Table 3). The MICs of two relapse isolates DR50054E and DR90031E were both 4-fold higher than those of their primary pairs, DR50054A and DR90031A (4 µg/ml versus 1 µg/ml for both pairs).

The BMD results for AMC confirmed two AMC-R isolates with MICs of 32/16 µg/ml (DR80110A and DR50054E) and two AMC-I isolates (DR40031A and DR80109A) with MICs of 16/8 µg/ml (Table 3). For the relapse pairs, only DR50054E was AMC-R with an increased MIC to 32/16 µg/ml compared with MIC 8/4 µg/ml of the primary isolate, DR50054A.

We also tested susceptibility to SXT which is used as a treatment option in some patients who are not responsive to β-lactams. Using BMD, we observed DR10212A was SXT-resistant (SXT-R) with an MIC of 4/76 µg/ml. In contrast, DR30013A, DR80110A,
Prevalence of antibiotic-resistant *B. pseudomallei* in northeast Thailand

To determine the prevalence of antibiotic resistance or intermediate resistance to clinically-relevant drugs used in treatment against *B. pseudomallei*, a combination of DD and BMD was used to interpret the results (Tables 2 and 3). Decreased susceptibility to one or more β-lactam antibiotics tested was observed for six primary isolates (6/1,304, 0.46%) consisting of CAZ-R (N=2), CAZ-I (N=1), MEM-LS (N=2), AMC-R (N=1) and AMC-I (N=2). These included one isolate (DR10212A) exhibiting both CAZ-R and MEM-LS (1/227, 0.44%) in Hospital A, one CAZ-R (1/26, 3.85%) isolate (DR30013A) in Hospital C and one AMC-I (1/140, 0.71%) isolate (DR40031A) in Hospital D. In Hospital H, of two isolates with reduced antibiotic susceptibility, one isolate (DR80110A) was AMC-R and CAZ-I (1/177, 0.57%), another one isolate (DR80109A) was AMC-I. One MEM-LS (1/107, 0.94%) primary isolate (DR90049A) was observed in Hospital I.

For relapse isolates, 2/13 (15.4%) had developed resistance to previously completed antibiotics. One relapse isolate (DR50054E) from Hospital E was CAZ-R, MEM-LS, AMC-R. The other relapse isolate (DR90031E) collected from Hospital I was MEM-LS.

Taken together, two multi-drug resistant (MDR) isolates: DR10212A (CAZ-R, MEM-LS, SXT-R) and DR50054E (CAZ-R, MEM-LS, AMC-R) were observed from this cohort.
Treatment history of patients infected with *B. pseudomallei* with decreased susceptibility to antibiotics

We evaluated the medical records of six patients who were infected with *B. pseudomallei* with decreased susceptibility to CAZ or MEM for clues to the development of antibiotic resistance (Table 4). Four patients were transferred from other hospitals to the study hospital sites so the initial treatment histories were incomplete. The overall mortality of melioidosis patients in this cohort was 33%. Two of six (33%) patients with CAZ or MEM non-susceptible isolates eventually died at day 40 (patient 1) and day 9 (patient 2) after admission. They had received CAZ or MEM for at least 9 days prior to isolation of *B. pseudomallei* with less susceptibility to the administered antibiotics. Patient 4 was admitted upon positive culture for *B. pseudomallei* (AMC-R) and had received unknown duration of AMC medication from referring hospital. A positive *B. pseudomallei* (MEM-LS) was cultured from blood specimen of patient 6. The patient had history of melioidosis two years ago and was not treated with MEM in the current admission. For the other two relapse cases, only patient 3 had received CAZ while patient 5 was treated with AMC instead of CAZ or MEM during their initial treatment course.

Genome analysis of genes associated with antibiotic resistance

Whole genomes of primary CAZ-R, MEM-LS and AMC-R isolates: DR10212A, DR30013A, DR80110A, DR90049A and relapse pair isolates: DR50054A-DR50054E and DR90031A-DR90031E were sequenced to define multilocus sequence types (ST) and searched for known and new mutations in β-lactam-resistance associated genes including
β-lactamase genes (penA, oxa), β-lactam target (PBP3), efflux pump systems and outer membrane porin (Table 5 and Table S2). We obtained > 90% sequence coverage of the K96243 reference genome for WGS of all isolates.

MLST of both DR10212A and DR30013A were ST10 which was identical to ST of K96243. MLST of DR80110A was ST531. DR50054A had the same ST288 as DR50054E relapse pair. DR90049A shared identical ST207 with relapse pair, DR90031A and DR90031E.

WGS analysis of β-lactam-resistance associated genes revealed a penA mutation in three isolates with CAZ-R phenotype (DR10212A, DR30013A, DR50054E) and a CAZ-S isolate (DR50054A) (Table 5). DR10212A and DR30013A had P167S at PenA omega loop also documented in CAZ-R isolates in previous works [20,22,27], DR50054A and DR50054E shared the same PenA mutation of I139M and T147A. DR80110A with CAZ-I, DR90049A and paired isolates DR90031A and DR90031E with MEM-LS bore wild-type (WT) PenA. We observed a SNP -71A>G at upstream promoter region of penA in DR30013A, DR80110A, DR50054A and DR50054E. In DR80110A, there was an additional -49A>C located upstream of penA. Analysis of oxa demonstrated that all of the isolates had Oxa-59 identical to K96243 (WT) except in DR50054A and DR50054E where an amino acid substitution at A268T occurred in both (Table 5).

No large deletion was observed in the β-lactam target PBP3 (BPSS1219), but we found two SNPs affecting PBP3 (I576V and A584T) in DR80110A and DR50054A-DR50054E pair isolates (Table 5). Interestingly, we observed in the MDR isolate DR50054E, a notable increase of read coverage approximately 6-fold involving 22-kb
regions (BPSS0944; NCBI new locus tag BPS_RS23855 to BPSS0960; NCBI new locus tag BPS_RS23950) containing *penA* and 19 other genes in chromosome 2 compared to the average genome coverage (Figure 2) (Table S3). This feature was not detected in the first episode isolate DR50054A and other resistant isolates.

We next examined the genetic alteration related to RND multi-drug efflux pumps, AmrAB-OprA, BpeAB-OprB and BpeEF-OprC. We observed that DR10212A had a deletion at *amrR* (V197del) of AmrAB-OprA efflux pump (Table 5). In strain DR90049A, we observed a non-synonymous SNP (R104C) in OprA and in-frame deletion of 6 amino acids (A202_R207del) in AmrR. Isolates DR50054A and DR50054E had a 11-bp deletion in *amrR* which conferred a frameshift at V222 (V222fs) and a SNP in *amrA* resulting in amino acid change, R116Q. Compared to primary isolate DR90031A, the relapse isolate DR90031E with MEM-LS phenotype had in-frame deletion of 63 amino acids from H92 to S154 shortening AmrR to 160 residues. Both DR90031A and DR90031E also contained R104C in OprA which was also observed in DR90049A.

For BpeAB-OprB efflux pump, except for DR90031A and DR90031E, P46S was found in BpeA of the other six isolates: DR10212A and DR30013A (CAZ-R), DR90049A (MEM-LS) and DR80110A (AMC-R) isolates and relapse pair isolates DR50054A and DR50054E (Table 5). P46S was the only variant identified in DR10212A. DR80110A shared BpeB N956K with DR50054A and DR50054E. Four SNPs resulting in amino acid substitution of A501S in OprB; P46S in BpeA; P709S and N956K in BpeB were common SNPs between the first isolate DR50054A and relapse pair DR50054E. Two SNPs in *bpeA*...
resulting in amino acid change, P390S and A414T were found in both DR90031A and DR90031E.

For BpeEF-OprC efflux pump, DR10212A encoded oprC, bpeE, bpeF, bpeT and bpeS WT genes. We observed amino acid alterations of V294A (7 isolates), T509A (5 isolates), V79A (3 isolates) and W154fs (1 isolate) in OprC, a A402T variant in BpeE (1 isolate) and in BpeS, K89R (1 isolate) and A311fs (1 isolate) (Table 5).

Gene encoding for outer membrane porin, Omp38 porin was also examined. A SNP in omp38 (R143P) was detected in DR80110A, DR50054A, DR50054E and DR90031A and DR90031E (Table 5).

Identification of genetic mechanisms for antibiotic resistance

To exclude unrelated resistance mechanisms, we next investigated whether SNPs in β-lactam-resistance associated genes and omp38 porin (Table 5) were present in genomes of CAZ-S, MEM-S and AMC-S isolates (N=697, Bioproject accession no. PRJEB25606) in our dataset. Most mutations were detected in antibiotic susceptible isolates. However, we observed some alterations involving only CAZ-R and MEM-LS strains including P167S in PenA; H92_S154del, V197del, A202_R207del in AmrR; W154fs in OprC and A311fs in BpeS. Further examination of genes affecting SXT susceptibility of SXT-R isolate, DR10212A revealed no mutation in folA, folM and folP which are involved in the tetrahydrofolate biosynthetic pathway (Table 5).

We hypothesized that alterations in PenA could have affected CAZ, MEM and AMC susceptibility in B. pseudomallei isolates presenting with MDR phenotype. Hence, in
this study, we focused on the evaluation of penA role in MDR strains DR10212A and DR50054E with decreased susceptibility towards β-lactam antibiotics. The SNPs found affecting penA were verified by Sanger Sequencing.

**Mutagenesis of penA in MDR strain DR10212A**

Both DR10212A and DR30013A had PenA P167S. DR30013A was CAZ-R while DR10212A was CAZ-R, MEM-LS and SXT-R (Table 3). PenA P167S has been reported previously to confer resistance to CAZ but not MEM [20,27] (Table S2) as observed in DR10212A. To evaluate the potential role of PenA on MEM susceptibility besides well-known CAZ, we proceeded with investigation on MDR strain DR10212A. First, we successfully created a penA deletion mutation in the parental strain, DR10212A, to observe the loss of resistance phenotype. DR10212AΔpenA::penA^K96243 was generated by complementation of wild-type K96243 penA into DR10212AΔpenA (Figure 3A).

PCR screening using two primer pairs to cover the whole penA sequence (Table S1) confirmed the generation of penA deletion and wild-type K96243 penA insertion. DR10212AΔpenA showed smaller penA fragment, 404 bp and 713 bp, upon deletion of the region containing the SNP compared to DR10212AΔpenA::penA^K96243 and wild-type K96243 penA, 858 bp and 1,167 bp (Figure 3B). DNA sequencing analysis confirmed the correct DR10212AΔpenA and DR10212AΔpenA::penA^K96243 sequences.

**Antibiotic susceptibility of DR10212A mutant and complemented strains**
We observed that DR10212A∆penA reduced MIC of CAZ by 64-fold from 128 (resistant) to 2 µg/ml (susceptible) and MEM by 4-fold from 8 (less susceptible) to 2 µg/ml (susceptible), respectively while maintaining the same for AMC (8/4 µg/ml, susceptible) and SXT (4/76 µg/ml, resistant) (Figure 3C). DR10212A∆penA::penAK96243 was CAZ-S and MEM MIC-S, showing MIC at 4 µg/ml, similar to penA contributing strain, K96243 and a MEM MIC-S, 2 µg/ml. Due to concerns about manipulating a possible bioterrorism agent, construction of resistant phenotype (P167S) by introduction of 517C>T from a resistant strain into original susceptible parental strain, K96243 was not conducted. Our data suggest that P167S in PenA is the mechanism responsible for CAZ-R and MEM-LS for DR10212A but not for SXT-R.

Growth curve analysis

Growth rate could be a determining factor for decreased β-lactam susceptibility as β-lactams act on metabolically-active cells. We observed a clear gap in the growth of DR10212A and its derivative mutants in LB broth which exhibited a longer lag-phase and slower growth compared to the other strains (Figure 4). Three strains (DR10212A, DR10212A∆penA and DR10212A∆penA::penAK96243) required an additional 2 h to reach mid-log phase before proceeding to penA transcriptional analysis and PenA β-lactamase activity in the next sections.

Quantification of penA transcript levels
To investigate whether the GDA event found in DR50054E is associated with elevated gene expression, qRT-PCR assay based on penA was employed in two culture conditions: LB and LB containing CAZ. The PenA wild-type and variant isolates were assessed on their penA expression profile. Indeed, DR50054E expressed a high level of penA transcripts (Figure 5A), suggesting that the overexpression was associated with GDA (Figure 2). Surprisingly, a CAZ-I and AMC-R isolate, DR80110A displayed relatively higher penA transcripts than the CAZ-R DR10212A. K96243 which served as the reference strain showed similar penA expression level as DR50054A. For CAZ induction analysis in CAZ-susceptible and resistant strains, we compared the expression of penA before and after CAZ treatment. There was no significant differential penA expression level observed between these two conditions (Figure 5A).

PenA β-lactamase activity

To validate the genetic mechanisms of resistance to β-lactams and penA expression levels, we determined PenA β-lactamase activity for DR10212A, DR80110A, DR50054A, DR50054E and DR10212A mutant derivative DR10212AΔpenA::penAK96243. Strain K96243 and DR10212AΔpenA were used as positive and negative control, respectively. Consistent with high penA expression level observed in DR50054E grown in LB, high PenA β-lactamase activity was also detected in DR50054E (Figure 5B). This observation further confirmed the elevated resistance due to GDA in DR50054E. Confirming the penA transcripts result, DR80110A had higher β-lactamase activity compared to K96243 and DR10212A.
Discussion

CAZ is commonly used for melioidosis treatment and MEM is reserved for severe cases in Thailand and many countries. Our data indicates that the prevalence of primary resistance to β-lactams is low: CAZ-R (0.15%) and MEM-LS (0.15%), and AMC-R (0.08%). Two of 13 relapse isolates exhibited decreased β-lactam susceptibility: CAZ-R (7.7%), MEM-LS (15.4%) and AMC-R (7.7%). All patients who exhibited CAZ-R (N=3) and AMC-R (N=2) had received CAZ and AMC prior or during their treatment course, respectively. Here, we described two clinical MDR strains of *B. pseudomallei* and two fatal cases involving PenA alterations. WGS analysis identified amino acid substitution of P167S in PenA and GDA events involving *penA*, the most prominent mechanisms of high CAZ MIC. In addition, we observed new mutations upstream of *penA* -49A>C relating to increased *penA* expression and in genes encoding for AmrAB-OprA and BpeEF-OprC efflux pumps systems that might be involved in decreased susceptibility to MEM.

DD is commonly used for antibiotic susceptibility testing (AST) in resource-limiting hospitals instead of laborious standard BMD, costly E-test and due to limited availability of automated systems [33-34]. Despite unavailability of interpretive guidelines for DD in CLSI, zone diameters for related organisms such as *Pseudomonas aeruginosa* and *Enterobacterales* [35] are applied with exception to SXT [12-14, 16, 33]. Interpretation of SXT susceptibility by disk diffusion could be misleading due to the difficulty to read the diffuse edges hence BMD or E-test is recommended [24, 34]. Due to its ease of use, E-test has been applied in laboratories instead of BMD [13, 15, 33, 37-39]. By DD method, MEM
and AMC resistance could be over-reported as previously described (Table S4). Our data in this study demonstrated misinterpretation by DD in Hospital A and Hospital H for AMC-I and MEM-R, respectively (Table 2) suggesting DD should be repeated and MIC-based BMD should be conducted as a confirmatory test for resistant or intermediate resistance results. Hospital I reported several IPM-R and MEM-R isolates. Investigations into the hospital reports pointed out that Vitek®2 AST cards misidentified B. pseudomallei as B. cepacia, Pseudomonas sp. or P. aeruginosa thus required an extra step to reconfirm B. pseudomallei identification by Vitek®2 GN ID card. It was noted that the MIC values generated by Vitek®2 AST cards for these isolates were within susceptible range of CLSI [36] but the automated interpretation of Vitek®2 system (advanced expert system) misinterpreted them as resistant. Therefore, the readers may refer to the CLSI guidelines to interpret the antibiotic susceptibility based on the MIC values when Vitek®2 AST cards are used for AST. Misidentification of resistant phenotypes could lead to a fatal outcome when an effective drug is not used. Strict monitoring in a timely manner of B. pseudomallei antibiotic susceptibility is required to avoid inappropriate administration of antibiotics.

The low number of isolates with primary CAZ and MEM resistance in this study was comparable to earlier reports from Thailand (CAZ-R, 0.1-1.5%; MEM-R, 2%) [12-14]. Primary CAZ resistance in Malaysia was reported at 0.6-2.4% [15-17] while Australia [37], Cambodia [38-39] and Vietnam [40] reported no primary resistance to CAZ or MEM (Table S4). In this study, we found two primary MEM-LS in Thailand which is worrisome. Previous studies based on E-test reported that all MEM susceptible isolates had MIC ≤ 2 µg/ml [13,15,37-38] but one report showed 2% primary MEM-R by DD in Thailand [12].
However, analysis by DD may be an over-representation and we used standard BMD as confirmation test in this study. We observed delayed MEM therapy in one fatal case (patient 2) involving a CAZ-R isolate, pointing out the importance of initial AST screening for appropriate antibiotic therapy. Unfortunately, another fatal melioidosis case (patient 1) was co-infected with MDR *A. baumannii*. The patient probably acquired CAZ-R and MEM-LS during therapy and showed no improvement upon AMC treatment despite DR10212A being AMC-S. In addition, DR10212A had a slower growth rate which could be a trade-off for fitness advantage to survive against β-lactams which kill metabolically active cells. A further study is required to investigate on the virulence of this strain.

All isolates were susceptible to IPM suggesting a favorable alternative and cross-resistance between meropenem and imipenem may not occur [30]. Yet, we noted that the absence of IPM-R could be due to infrequent IPM use therefore no antibiotic selection pressure. We also recorded that for two relapse cases with decreased susceptibility to the originally susceptible CAZ or MEM or AMC, both patients received either IV AMC plus oral AMC and SXT or IV CAZ plus oral AMC treatments during first episode. The use of second-line AMC [7] as treatment may be ineffective for bacterial clearance. Moreover, MEM-LS isolates were recovered from both patients 3 and 5 despite never being treated with MEM in their initial treatment course, also postulating the possible occurrence of cross-resistance to AMC, ceftriaxone or TB drugs such as azithromycin and streptomycin received during the therapy.

Our study indicated that both previous described PenA P167S [20,22] and GDA [21,26] events are common CAZ-R conferring mechanisms of *B. pseudomallei* isolated
from patients during and after treatment with CAZ (Table S2). In contrast to PenA T147A shown to confer resistance to both AMC and IPM [25], we shared similar observation with Sarovich et al that T147A by itself was not associated with AMC and IPM resistance [30]. A search in our whole genome database indicated that both PenA I139M and T147A observed in relapse pairs, DR50054A and DR50054E are commonly found in B. pseudomallei isolates susceptible to CAZ, MEM and AMC, therefore these mutations may not be significantly associated with resistance in DR50054E.

No other mutation besides two SNPs at position 49 (-49A>C) and 71 (-71A>G) upstream of penA, were observed in CAZ-I isolate, DR80110A. Since -71A>G is commonly observed in CAZ-S B. pseudomallei isolates in our dataset, SNP 49A>C could be the factor leading to increased penA expression in strain DR80110A. This mutation could have interfered with the terminator function of penA transcriptional terminator (Rho-independent TERM264) situated between nucleotides -25 and -73 upstream of penA [41]. Although infrequent, this mutation was also detected in 1.4% of CAZ-S isolates in our dataset, suggesting that this SNP, -49A>C probably reduced bacterial susceptibility to CAZ but insufficient to result in CAZ resistance.

PBP3 deletion is associated with CAZ-R phenotype and growth defect [19]. Although DR50054A, DR50054E and DR80110A had I576V and A584T amino acid changes in PBP3, these mutations were also observed in CAZ-S isolates hence unlikely to be associated with CAZ-R. We noted that DR50054A, DR50054E and DR80110A were able to grow normally in LB comparable to K96243 (Figure 4).
TetR-type regulator gene, amrR acts as the repressor of AmrAB-OprA efflux pump and is responsible for efflux pump overexpression and elevated MEM MIC [30-31]. The new amrR mutation includes deletions at two different positions leading to H92_S154del and A202_R207del in strains DR90049A and DR90031E are possibly associated with MEM-LS phenotype. LysR-type regulators, bpeS and bpeT act as transcriptional activators of BpeEF-OprC efflux pump, together with tetra-hydrofolate biosynthetic pathway: folA, folM and folP contribute to SXT-R phenotype [21,32]. However, we observed no alteration of these genes in SXT-R isolate DR10212A. Instead, new frameshift mutations in oprC (W154fs) and bpeS (A311fs) was observed in MEM-LS DR90049A, suggesting possible connection between MEM-LS phenotype and BpeEF-OprC efflux pump [24,30]. The mechanism related to the SXT-R profile in DR10212A could be due to AmrAB-OprA V197del or mechanisms other than BpeEF-OprC efflux pump and tetra-hydrofolate biosynthetic pathway [30,32].

In addition to CAZ-R, we associated the decreased MEM susceptibility in DR10212A with PenA P167S as our data showed that upon deletion or replacement of penA in DR10212A (128 µg/ml) with wild-type penA^K96243, both the MIC of CAZ and MEM dropped into the susceptible category. The CAZ MIC reduced by 64-fold and 32-fold in DR10212AΔpenA (2 µg/ml) and DR10212AΔpenA::penA^K96243 (4 µg/ml), respectively. The MEM MIC reduced by 4-fold to 2 µg/ml in both mutants. Both AMC and SXT MICs were unaltered following penA replacement. Taken together, the data suggested that PenA P167S was responsible for the CAZ-R and MEM-LS phenotypes in DR10212A. Our observation was in concordance with previous reports that PenA P167S confers CAZ-R
phenotype [20,22,27,44] however differed in the second notion that P167S also resulted in reduced susceptibility to MEM. To start with, the parental MDR strain DR10212A had higher MIC of CAZ and MEM at 128 µg/ml and 8 µg/ml compared to 64 µg/ml and 1 µg/ml of DR30013A and a clinical isolate from P45 (Australia), both containing PenA P167S [20]. The apparently higher MIC value might be due to the relatively slower growth of DR10212A compared to DR30013A. Another Thai clinical isolate, 316c bearing PenA P167S variant also had CAZ MIC at 64 µg/ml [22]. Despite lower MIC of CAZ at ≥32 µg/ml, Ho et al demonstrated that strain BPLH-1-2 with PenA P167S generated upon selection on increasing CAZ concentrations had elevated MEM MIC by 2-fold to 4 µg/ml (MEM-LS) compared to wild-type strain, BPLH-1 [42]. However, a laboratory generated PenA P167S mutant, Bp82.5 exhibited increased MIC of CAZ from 3 µg/ml to 16-24 µg/ml and unchanged MIC of MEM at 0.5-0.75 µg/ml compared to parental strain, Bp82 [27]. Our experiment differed from Rholl et al. that we replaced the PenA P167S with wild-type PenA K96243 in parental strain, DR10212A instead of introducing mutation into the laboratory strain Bp82 [27].

Occurrence of GDA event was previously reported in strains MSHR5654 and isogenic pairs MSHR8441 and MSHR8442 from patients in Australia [21] and Bp5041c from patient in Thailand [26]. GDA was observed in Bp5041c isolated after 15 days of CAZ therapy [26]. Yet, in our study, DR50054E which presented with GDA was isolated 341 days after completed CAZ therapy. Similarly, MSHR5654 with GDA was isolated approximately 25 months after completion of CAZ and MEM therapy [21]. In addition to
GDA event involving penA region, MSHR5654 also had PenA C69Y (Table S2) associated with high CAZ-R [18,21,23] and a BpeT T314fs conferring SXT-R [21,31]. In DR50054A and DR50054E (CAZ-R, MEM-LS, AMC-R), there were no other gene mutations observed for oxa-59, PBP3 or efflux pumps differentiating this pair except for GDA event.

Our study confirmed that the presence of CAZ in culture medium for 2 h had no effect in the CAZ susceptibility and penA expression level compared with isolates cultured in CAZ untreated medium, consistent with Rholl et al [27].

Alterations involving GDA in CAZ-R isolate, DR50054E and -49A>C upstream of penA in CAZ-I isolate, DR80110A exhibited increased penA expression level and β-lactamase activity. However, the penA expression level and β-lactamase activity do not correlate well with CAZ MIC level observed in DR10212A and DR50054A. A lower penA expression level and β-lactamase activity were observed in DR10212A despite showing high CAZ MIC. It is likely that in DR10212A, P167S substitution at omega loop of PenA led to increased affinity for CAZ compared to wild-type PenA resulting in high CAZ resistance [22,42]. It was also observed in CTX-M-14 and CTX-M-19 of extended spectrum β-lactamase (ESBL) Enterobacteriaceae harboring P167S that the mutation affects the binding and interaction with the aminothiazole ring of CAZ resulting in enhanced CAZ hydrolysis [43-44].

PenA alterations could relate to decreased CAZ and probably MEM susceptibility. Mutation detection focusing on PenA SNPs including promoter region would be valuable in clinical settings, prompting on the change to alternative drugs such as IPM to alleviate the possibility of treatment failure due to ineffective antibiotics. For instance, rapid
identification of PenA P167S using real-time PCR and RNA-based triplex qPCR assay targeting upregulation of AmrAB-OprA, BpeEF-OprC and BpeAB-OprB efflux pumps have been developed by Sarovich et al. and might be useful [20,31].

The determinant for AMC resistance is still unclear. Further assessment on other mechanisms besides enzymatic inactivation by PenA in this study would be beneficial. Currently, we are in the progress of evaluating the putative mutations involving efflux pumps mechanism: amrR (H92_S154del, V197del, A202_R207del), oprC (W154fs) and bpeS (A311fs), which possibly give rise to MEM-LS and SXT-R.

Conclusion

The low prevalence of *B. pseudomallei* isolates with resistance to β-lactams *in vitro* suggest the appropriateness of CAZ and carbapenem treatment regimens. Alterations affecting penA including SNPs, gene duplication events, and the upstream promoter region are currently the major determinants corresponding to decreased CAZ susceptibilities. In addition to efflux pump system, PenA may extend its role to affect MEM susceptibilities. Further validation into the efflux pumps mechanism observed in MEM-LS and SXT-R isolates will be required to ascertain their phenotypes. The mortality rate of melioidosis still remains high raising questions about the association of other determinants with regards to patient outcomes, however, the unfilled gap leading to treatment failure requires further investigation.

Materials and methods
Ethical approval
The Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (approval number, MUTM2015-002-05) has approved this study and the consent procedure. Written informed consent was obtained from all participants or their representatives.

Biosafety approval
This study has been approved by Institutional Biosafety Committee, Faculty of Tropical Medicine, Mahidol University (approval number, FTM-IBC-21-01).

Bacterial isolates
A total of 1,317 clinical B. pseudomallei isolates were obtained from 1,304 patients in nine hospitals in northeast Thailand between July 2015 and December 2018. These included 1,304 primary isolates and 13 isolates from relapse cases. Primary and relapse isolates were denoted as A and E, respectively at the end of strain name. The hospitals and number of isolates were as follows: Hospital A (N=227), Hospital B (N=89), Hospital C (N=26), Hospital D (N=140), Hospital E (N=226), Hospital F (N=198), Hospital G (N=127), Hospital H (N=177) and Hospital I (N=107). The isolates were identified as B. pseudomallei at the hospitals and confirmed at the Faculty of Tropical Medicine, Mahidol University (FTM) by a specific exopolysaccharide-monoclonal-based latex agglutination assay [45] and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) [46]. The source and site of the specimens varied from blood (N=940); respiratory
secretions (N=133), body fluid (N=44), pus (N=164), urine (N=26), wound swab (N=3) and tissue (N=7) (Table 1). The bacteria were stored in tryptic soy broth (TSB) with 20% glycerol at -80°C until use. All \textit{B. pseudomallei} cultures were performed in a biosafety level 3 laboratory (BSL3).

\textbf{Determination of antibiotic susceptibility}

Four different procedures were used by the nine hospitals for antibiotic susceptibility testing (AST) (Figure 1). Hospitals A, B, E and H used DD only; Hospital C, Hospital F and G used a combination of DD and E-test; Hospital D used disk DD, BMD and BD Phoenix\textsuperscript{TM} automated microbiology system (BD Diagnostics Systems); and Hospital I used Vitek\textsuperscript{®}2 system (bioMérieux). At FTM, we performed AST for clinical \textit{B. pseudomallei} isolates that were either not performed or reported as intermediate resistant or resistant by the hospitals. \textit{B. pseudomallei} were cultured on Mueller-Hinton agar (MHA) (Oxoid) or cation-adjusted Mueller-Hinton broth (CAMHB) (Sigma). Antibiotic susceptibility was tested by DD and confirmed with BMD. The antibiotic discs used were CAZ, IPM, MEM and AMC (Oxoid). The antibiotic concentration ranges used for BMD were as follows: CAZ (0.25 to 256 µg/ml), MEM (0.03 to 32 µg/ml), AMC (0.06/0.03 to 64/32 µg/ml), and SXT (0.03/0.57 to 32/608 µg/ml) (Sigma). Standard MIC panels were prepared with CAMHB containing the serial drug dilutions with a final volume of 100 µl per well.

Bacterial suspensions in 0.85% sodium chloride were prepared from 18 h culture on Columbia agar (CA) to achieve a target concentration of approximately 1 X 10\textsuperscript{8} CFU/ml.
for DD and 5 X 10^5 CFU/ml for BMD. The results were read after 18 h incubation at 37°C. CLSI guidelines were used for the interpretation of both DD and BMD. The threshold zone sizes of *Enterobacterales* and *Pseudomonas aeruginosa* was applied as reference for DD [35]. For BMD, *Escherichia coli* (ATCC 25922), *E. coli* (ATCC 35218), *P. aeruginosa* (ATCC 27853) and *B. pseudomallei* K96243 were used as controls. MIC reference categories for *B. pseudomallei* are available for CAZ, AMC and SXT [36] except for MEM, hence we considered the epidemiological cut-off value (ECOFF) of >2 µg/ml as the breakpoint to differentiate strains with decreased MEM susceptibility from the wild-type [47].

Whole genome sequencing

The whole genomes of *B. pseudomallei* isolates were sequenced using Ion Torrent or Illumina platform (Illumina MiSeq or HiSeq2000) at the Center for Medical Genomics, Faculty of Medicine, Ramathibodi Hospital, Bangkok, Thailand and the Wellcome Sanger Institute at UK. Briefly, the genomic DNA was extracted from 1.5 ml overnight bacterial culture in LB broth using QIAmp DNA mini kit (Qiagen, Germany). The DNA libraries were prepared for 150-base-read with Ion Xpress Plus Fragment Library Kit (Life Technologies) for Ion Torrent system and 75 or 250-base-paired-end-read libraries for Illumina system. The short reads produced from both platforms were mapped to the reference *B. pseudomallei*, K96243 genome (NC_006350.1, NC_006351.1) using CLC genomic workbench version 20.0 (CLC Bio-Qiagen). Multilocus sequence type was analyzed using https://pubmlst.org/.
Antibiotic-resistance conferring genes

Whole genome searches were performed on the antibiotic-resistant associated genes described previously [5,48] including β-lactamase genes: penA (BPSS0946; BPS_RS23870) and oxa (BPSS1997; BPS_RS29690); β-lactam drug target, PBP3 (BPSS1219; BPS_RS25365); RND multi-drug efflux systems and respective regulators: AmrAB-OprA (BPSL1802-BPSL1804; BPS_RS09570-RS09580), amrR (BPSL1805, BPS_RS09585), BpeAB-OprB (BPSL0814-BPSL0816; BPS_RS04290-RS04300), bpeR (BPSL0812, BPS_RS04280) and BpeEF-OprC (BPSS0292-BPSS0294; BPS_RS20225-RS20235), bpeT (BPSS0290, BPS_RS20215), bpeS (BPSL0731, BPS_RS03845); tetrahydrofolate biosynthesis pathways: folA (BPSL2476; BPS_RS13300), folM (BPSS0039; BPS_RS18745) and folP (BPSL1357; BPS_RS07190); and outer membrane protein Omp38 (BPSS0879; BPS_RS23505). Briefly, DNA sequences were aligned and annotated with the reference to observe for single nucleotide polymorphisms (SNPs), insertion/deletions (indels) and gene duplication and amplification event (GDA). Other mutations in the antibiotic targets, efflux pumps and tetrahydrofolate biosynthesis pathways were explored on bacterial genomes. The variants involving alterations in amino acid were analysed and compared with susceptible isolates.

DNA sequencing
The mutations involving penA sequences were verified by Sanger sequencing (SolGent, South Korea) using primer PenA1 and primer PenA2 pairs (Table S1). The primers were designed using Primer-BLAST (https://ncbi.nlm.nih.gov/tools/primer-blast).

Construction of penA deletion mutant and complemented strains

A penA mutant and complemented B. pseudomallei on strain DR10212A were constructed based on pEXKm5 gene replacement as previously described [49-50]. Briefly, two fragment sequences containing penA<sup>DR10212A</sup> deletion and insertion of penA<sup>K96243</sup>, respectively were designed. These regions were flanked by homology sequences upstream and downstream of penA in DR10212A to allow for homologous recombination to integrate the desired mutations into the chromosome. A stop codon was included in the deletion fragment to generate a non-functional PenA. The penA deletion (pUC57ΔpenA<sup>DR10212A</sup>) and insertion (pCCI-4k::penA<sup>K96243</sup>) fragments were synthesized (Genscript, USA). The plasmid vectors were double-digested with XhoI and EcoRI (Takara, Japan). The correct fragment size was excised from gel and purified using a Qiaquick purification kit (Qiagen, Germany) and ligated to pEXKm5 using a Ligation Mighty Mix (Takara, Japan). The plasmid was later transformed into E. coli, DH5α followed by RHO3. White kanamycin-resistant colonies were selected on LB agar with 35 µg/ml kanamycin containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Promega, Italy). The colonies with constructed pEXKm5 were verified by correct fragment size by PCR using PenA-muta primers for the amplification of the internal region of penA (Table S1). Constructed pEXKm5-containing RHO3 were later conjugated with B. pseudomallei on a nitrocellulose
membrane on LB agar containing 400 µg/ml diaminopimelic acid (DAP). The merodiploid clones were selected and visualized as blue colonies on LB agar containing 1000 µg/ml kanamycin and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) (EMD Millipore, Switzerland). The correct fragment size was confirmed by PCR using primers described in Table S1. The clones upon sacB-mediated counterselection on sucrose containing yeast-extract tryptone agar were examined for kanamycin sensitivity. The penA sequences of kanamycin-sensitive mutants were verified by Sanger sequencing (SolGent, South Korea).

**Bacterial growth curve analysis**

Growth curve analysis was performed on *B. pseudomallei* isolates to assess the growth rate of antibiotic resistant and laboratory-constructed mutant strains. Strain K96243 was used as the reference and the initial isolate, strain DR50054A was included for comparison with relapse isolate, strain DR50054E. Briefly, one colony of *B. pseudomallei* was suspended in 3 ml LB broth and incubated at 37°C with shaking at 200 rpm overnight. The bacteria were then inoculated at a dilution of 1:100 into 5 ml of LB broth to obtain bacterial concentration at approximately 1 X 10^6 CFU/ml and incubated at 37°C with shaking at 200 rpm for 10 h. The OD 600 nm value was recorded for every 2 h interval.

**RNA extraction and transcripts quantification**

Two-step reverse-transcription real-time PCR (RT-PCR) was used to compare penA transcriptions of *B. pseudomallei* strains grown in LB broth with and without CAZ as previously described [27]. Bacteria were harvested upon growth in LB broth at 37°C for 5
to 7 h upon reaching mid-log phase. For the antibiotic-induced condition, the bacteria were
further incubated in LB medium with 32 µg/ml CAZ for additional 2 h at 37°C with shaking
at 200 rpm. RNA was extracted using Trizol reagent (Invitrogen) followed by Turbo®
DNase treatment (Invitrogen). Genomic DNA removal was evaluated by absence of 16S
rDNA gene using a SYBR Green-based qPCR. The total RNA was converted to cDNA by
iScript Reverse Transcription Supermix (Bio-Rad) according to manufacturer’s instruction.
The quantitative assessment on the penA transcriptions was conducted in duplicate using
iTaq® Universal SYBR Green Supermix (Bio-Rad) on CFX96 Touch Real-Time PCR
Detection System (Bio-Rad). The RT-PCR conditions were performed as follows: initial
denaturation at 95 °C for 5 minutes followed by 45 cycles of denaturation at 95 °C for 15
seconds; annealing and extension at 54 °C for 30 seconds. After amplification, melting
curve analysis was conducted by increasing the annealing temperature by 0.1 °C per step
from 65 °C to 95 °C. The primer-pair used were PenA4 (Table S1). 16S rDNA gene was
used as the housekeeping control for data normalization [51]. The normalized expression
levels were calculated using the formula: 2^{-ΔCq}, where ΔCq=(Cq_{penA} – Cq_{16S}). Student t-test
was used to compare the differences in quantitative data between the bacteria grown in LB
with CAZ versus bacteria grown in LB. The groups were considered statistically significant
at p<0.05. All quantitative data are shown as mean ± standard deviation (SD).

**β-lactamase assay**

A β-lactamase assay was performed to quantify the β-lactamase activity in bacterial
cultures as previously described [26]. This is based on β-lactamase activity produced from
bacteria via hydrolysis of a chromogenic cephalosporin, nitrocefin. Briefly, 20 μl of bacterial culture grown in LB medium to mid-log phase (OD 600 nm =0.5-0.6) was incubated with 0.2 ml of 0.5 mg/ml nitrocefin (Merck, USA) in 100 mM NaPO4 at pH 7 at 37°C in 96-well plate. The absorbance was read at OD 486 nm with Tecan Sunrise™ microplate reader (Tecan, Switzerland). A mutant with penA deletion, DR10212AΔpenA was used as the negative control to eliminate the possible background activity of nitrocefin substrate. The ΔA486/min was calculated from the linear portion of the curve and PenA activity units calculated as (ΔA486/10^6 cells x min) x 10^5.

Data availability
The GenBank accession numbers for strain K96243 are NC_006350.1, NC_006351.1. The accession numbers for the resistant and susceptible isolates are available under Bioproject accession number PRJEB25606. The ENA and GenBank accession numbers for strains DR10212A, DR50054E, DR90049A, DR30013A, DR50054A, DR80110A, DR90031A and DR90031E are SRR12710798, SRR12710797, SRR12710796, ERA1581689, ERA1581823, ERA1582124, ERA1582153, SRR12710795, respectively. Sequences associated with specific PubMLST allele numbers can be retrieved from https://pubmlst.org/

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NC and TEW designed the study; SHYF, PE, NSP, ST conducted the experiments; RP, ST, CC, RS, ET, SCS, TH, SCN, KT, SCY, CM, NSS, WS, NB and PC acquired data; SHYF, RP, ST and NC analysed data; ET, SCS, TH, SCN, KT, SCY, CM, NSS, WS, NB, PC, NJD, WC, GL and NC provided samples or reagents or facilities; SHYF, TEW and NC wrote the manuscript.

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Figures legends

Figure 1. Flow chart for identification and antibiotic susceptibility testing of *Burkholderia pseudomallei* at nine hospitals in northeast Thailand and Faculty of Tropical Medicine, Mahidol University (FTM).

Figure 2. Gene duplication and amplification (GDA) in clinical *Burkholderia pseudomallei* strain DR50054E.

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Table legends

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Figure 1. Flow chart for identification and antibiotic susceptibility testing of *Burkholderia pseudomallei* at nine hospitals in northeast Thailand and Faculty of Tropical Medicine, Mahidol University (FTM)

MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry
Figure 2. Gene duplication and amplification (GDA) in clinical *Burkholderia pseudomallei* strain DR50054E. The 22-kb amplified regions in chromosome 2 of relapse strain, DR50054E, involve 20 genes from *BPSS0944* to *BPSS0960* (red line) (Table S3) including *penA* (purple line). The average sequence read coverage of this GDA region is approximately 6-fold higher than the average genome coverage.
Figure 3. Construction of penA mutants in *Burkholderia pseudomallei* strain DR10212A. (A) The construction of DR10212AΔpenA and DR10212ΔpenA::penA<sub>K96243</sub>. penA is also known as *BPSS0946* (*BPS_RS23870*) (pink). The gene located upstream of penA is *BPSS0945*; *BPS_RS23865* (green). The downstream genes are NCBI recently annotated hypothetical gene, *BPS_RS23875* (dark blue) followed by *BPSS0948*; *BPS_RS238800* (yellow). In step 1, penA encoding P167S (light blue) was knocked-out from the parental strain DR10212A and replaced with wild-type penA from K96243 in step 2 to produce DR10212AΔpenA::penA<sub>K96243</sub>. Briefly, fragments containing the desired penA deletion and insertion sequences were synthesized. The plasmid vectors containing the cloned regions of homology allowed the exchange and integration of desired fragments into the
chromosome by homologous recombination between cloned and chromosomal sequence. Multiple counter-selection markers were applied using kanamycin and sucrose to finally obtain a desired clone. (B) The correct size of the products was confirmed with PCR using primer penA_muta (left) and penA1 (right). M, 100bp plus ladder; 1, DR10212AΔpenA; 2, DR10212AΔpenA::penAK96243; 3, K96243 and 4, sterile water. The gels were sliced for labelling purposes. (C) MIC of CAZ for DR10212A (orange), DR10212AΔpenA (black) and DR10212AΔpenA::penA K96243 (purple) was 128 µg/ml (R), 2 µg/ml (S) and 4 µg/ml (S), respectively. After complementation with wild-type penA from K96243, the MIC of MEM dropped from 8 µg/ml (DR10212A, LS) to 2 µg/ml (DR10212AΔpenA and DR10212AΔpenA::penA K96243, S). The MIC of all isolates towards AMC and SXT remained unchanged showing AMC-S and SXT-R phenotypes.
Figure 4. Growth curves of *Burkholderia pseudomallei* isolates cultured in LB.
Figure 5. Expression of penA and β-lactamase activity of clinical Burkholderia pseudomallei isolates and laboratory-generated penA mutants. (A) qRT-PCR analysis was performed to compare the level of penA expressed. The strains used were K96243 (CAZ-S), DR10212A (CAZ-R), DR80110A (CAZ-I) and primary-relapse pairs: DR50054A (CAZ-S) and DR50054E (CAZ-R). Briefly, bacteria cells were grown to mid-log phase in LB broth. Equal portions of the cultures remained untreated or were treated with subinhibitory concentration at 32 µg/ml ceftazidime. Total RNA extraction was conducted on bacterial cells at mid-log phase. The penA gene expression level shown were after normalization with 16S cDNA. The error bars indicated the standard deviation between two biological replicates. (B) PenA activity for isolates DR10212A, DR80110A, DR50054A, DR50054E and DR10212AΔpenA::penAK96243 grown in LB. The enzyme activity was analyzed using nitrocefin as reporter substrate. The data was analyzed based on two individual experiments conducted on separate days. The positive and negative control were
K96243 and DR10212AΔpenA respectively. PenA β-lactamase activity units were derived by subtracting the activity observed with DR10212AΔpenA.
Table 1  Number of *Burkholderia pseudomallei* isolates and clinical specimens from melioidosis patients at nine hospitals in northeast Thailand

| Hospital code | Number of isolates | Clinical specimens |
|---------------|--------------------|--------------------|
|               | Blood | Respiratory secretion | Body fluid | Pus | Urine | Wound swab | Tissue |
| A             | 227   | 185 | 11 | 16 | 14 | 0 | 1 | 0 |
| B             | 89    | 74  | 9  | 1  | 5  | 0 | 0 | 0 |
| C             | 26    | 12  | 0  | 3  | 7  | 0 | 1 | 3 |
| D             | 140   | 96  | 20 | 3  | 18 | 3 | 0 | 0 |
| E             | 226   | 149 | 30 | 5  | 35 | 7 | 0 | 0 |
| F             | 198   | 135 | 26 | 6  | 23 | 6 | 0 | 2 |
| G             | 127   | 86  | 10 | 3  | 23 | 5 | 0 | 0 |
| H             | 177   | 128 | 16 | 5  | 23 | 3 | 1 | 1 |
| I             | 107   | 75  | 11 | 2  | 16 | 2 | 0 | 1 |
| **Total**     | 1,317 | 940 | 133 | 44 | 164 | 26 | 3 | 7 |

1 Body fluids from peritoneum, cerebrospinal, pericardium, pleural cavity, liver, synovial joints, elbow, joint, ankle, knee or bile

2 Pus from liver, kidney, neck, shoulder, arm, leg, ankle, knee or foot

3 Tissue from aneurysmal wall, lymph node or unknown origin
Table 2 Antibiotic susceptibility interpretation of initial screening and validation of non-susceptible *B. pseudomallei* isolates

| Hospital code and *B. pseudomallei* isolate | Isolate | Clinical specimens | Antibiotic   | Initial screen¹ | Validation² | Hospital FTM (DD) | Validation FTM (BMD) |
|-------------------------------------------|---------|--------------------|--------------|-----------------|-------------|-------------------|----------------------|
| DR10212A Primary                        | Pleural fluid | CAZ, MEM         | R            | R               | R           | R                 | LS                   |
| DR10118A Primary                        | Blood    | AMC               | I            | S               | S           | S                 |                      |
| DR10120A Primary                        | Blood    | AMC               | I            | S               | S           | S                 |                      |
| DR30013A Primary                        | Blood    | CAZ               | NA           | R               | R           | R                 |                      |
| DR40031A Primary                        | Blood    | CAZ, AMC          | I            | S               | S           | S                 | I                    |
| DR50054E Relapse                        | Sputum   | AMC               | ND           | R               | R           | R                 | R                   |
| DR80176A Primary                        | Sputum   | MEM               | R            | S               | S           | S                 |                      |
| DR80177A Primary                        | Sputum   | MEM               | R            | S               | S           | S                 |                      |
| DR80109A Primary                        | Sputum   | AMC               | ND           | I               | I           | I                 |                      |
| DR80110A Primary                        | Urine    | AMC               | ND           | R               | R           | R                 |                      |
| DR90087A Primary                        | Blood    | CAZ, MEM, IPM    | R            | S               | S           | S                 | S                   |
| DR90049A Primary                        | Blood    | MEM               | I            | S               | LS          |                   |                      |
| DR90003A Primary                        | Blood    | IPM               | R            | S               | S           | S                 |                      |
| DR90026A Primary                        | Sputum   | IPM               | R            | S               | S           | S                 |                      |
| DR90036A Primary                        | Blood    | IPM               | R            | S               | S           | S                 |                      |
| DR90045A Primary                        | Sputum   | IPM               | R            | S               | S           | S                 |                      |
| DR90076A Primary                        | Body fluid | IPM             | R            | S               | S           | S                 |                      |
| DR90031E Relapse                        | Pus      | MEM               | NA           | I               | LS          |                   |                      |

A, primary isolate; E, relapse isolate; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; AMC, amoxicillin-clavulanic acid; S, susceptible; I, intermediate; R, resistant.
LS, less susceptible for meropenem; ND, not done; NA, not available; FTM, Faculty of Tropical Medicine, Mahidol University; DD, disk diffusion test

1 Antibiotic susceptibility testing screening by hospitals and FTM as described in Figure 1.

2 Validation by broth microdilution method (BMD) by FTM.
Table 3 Antibiotic susceptibility profile of *Burkholderia pseudomallei* isolates analyzed in this study and CLSI susceptibility breakpoints for antibiotics

| Strain   | Isolate | CAZ (µg/ml) | MEM (µg/ml) | AMC (µg/ml) | SXT (µg/ml) |
|----------|---------|-------------|-------------|-------------|-------------|
| K96243   | Laboratory | 4 (S) | 1 (S) | 8/4 (S) | 2/38 (S) |
| DR10212A | Primary | 128 (R) | 8 (LS) | 8/4 (S) | 4/76 (R) |
| DR30013A | Primary | 64 (R) | 1 (S) | 2/1 (S) | 2/38 (S) |
| DR80110A | Primary | 16 (I) | 2 (S) | 32/16 (R) | 0.5/9.5 (S) |
| DR90049A | Primary | 2 (S) | 4 (LS) | 8/4 (S) | 0.5/9.5 (S) |
| DR40031A | Primary | 8 (S) | 1 (S) | 16/8 (I) | 1/19 (S) |
| DR80109A | Primary | 1 (S) | 1 (S) | 16/8 (I) | 0.5/9.5 (S) |
| DR50054A | Primary | 2 (S) | 1 (S) | 8/4 (S) | 1/19 (S) |
| DR50054E | Relapse | 64 (R) | 4 (LS) | 32/16 (R) | 0.5/9.5 (S) |
| DR90031A | Primary | 2 (S) | 1 (S) | 4/2 (S) | 0.25/4.75 (S) |
| DR90031E | Relapse | 2 (S) | 4 (LS) | 4/2 (S) | 0.25/4.75 (S) |

Interpretation

| CLSI susceptibility breakpoint (µg/ml) | CAZ | MEM | AMC | SXT |
|--------------------------------------|-----|-----|-----|-----|
| S                                    | ≤8  | NA  | ≤8/4| ≤2/38|
| I                                    | 16  | NA  | 16/8| NA  |
| R                                    | ≥32 | NA  | ≥32/16| ≥4/76|

CAZ, ceftazidime; MEM, meropenem; AMC, amoxicillin-clavulanic acid; SXT, trimethoprim-sulfamethoxazole; R, resistant; LS, less susceptible; S, susceptible; NA, not available
MICs for each isolate were determined after 18 hours incubation at 37°C and interpretation as recommended in Clinical Laboratory and Standards Institute (CLSI) M45, 2016 [36]. Interpretation for MEM is not available for B. pseudomallei in CLSI [36]. In this study, MEM-LS refers to isolates with MIC >2 µg/ml [47].
Table 4. Details of six patients with *B. pseudomallei* isolates resistant or less susceptible to β-lactam antibiotics.

| Patient No. and study hospital | Clinical presentation and medical history | Day and type of specimen collection after admission (Day) | Isolate ID (Antibiotic susceptibility result) | Treatment received after admission in study hospitals | Patient status |
|-------------------------------|------------------------------------------|--------------------------------------------------------|-----------------------------------------------|---------------------------------------------------|----------------|
| 1* (Hospital A)               | Fever, underlying diabetes mellitus, history of left lobectomy, required mechanical ventilation, empyema thoracis and broncho pleural fistula (Consecutive drainages done on Day 8, 9, 23 and 36). Co-infected with MDR *Acinetobacter baumannii*. He was provisionally treated as melioidosis and had received an unknown duration of CAZ in a referral hospital. | 9 (pleural fluid) | DR10212A (CAZ-R, MEM-LS, SXT-R) | MEM AMC 0 21 13 15 39 22 | Died at day 40 |
| 2* (Hospital C)               | Poorly controlled type 2 diabetes mellitus and fever. He had received CTR (3 days) and CAZ (9 days) from a referral hospital. | 0 (blood) | DR30013A (CAZ-R) | CAZ SXT CST MEM VAN 0 4 4 7 5 9 5 8 9 | Died at day 9 |
| 3 (Hospital E)                | Underlying TB with ongoing treatment, hemoptysis, chronic obstructive lung disease and renal impairment. Relapsed infection | 1 (sputum) | DR50054A (S) | CAZ AZM STR SXT NA NA NA 0 4 1 21 19 | Discharged at day 22 with home oral AMC Relapse |
|   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|
| 4*  | Dysuria, renal calculi and he had received unknown duration of AMC from a referral hospital. | 354 days after the first episode (sputum) | DR50054E (CAZ-R, MEM-LS, AMC-R) | CAZ | SXT | Discharged at day 13 with home oral SXT |
| 5*  | Fever, history of foot wound exposure to soil and carotid space abscess. He was diagnosed with hypertensive emergency, cardiomegaly and pulmonary congestion. | 1 (blood) | DR90031A (S) | CTR | AMC | Discharged at day 6 with home oral SXT and AMC |
| 6   | History of melioidosis two years ago, fever and splenic abscess. | 1 (blood) | DR90049A (MEM-LS) | CTR | DOX | Discharged at day 15 with home oral SXT |

MDR, multi-drug resistant; TB, tuberculosis; MEM, meropenem; AMC, amoxicillin-clavulanic acid; CFZ, ceftazidime; AZM, azithromycin; SXT, trimethoprim-sulfamethoxazole; CST, colistin; VAN, vancomycin; STR, streptomycin; CTR, ceftriaxone; DOX, doxycycline; S, susceptible; LS, less susceptible; R, resistant; NA, not available; *, transferred from referring hospitals.
Table 5 Whole genome analysis on antibiotic-resistant associated genes in B. pseudomallei isolates. Mutations were amino acid changes due to single nucleotide polymorphism, deletion and frameshift mutation observed in B. pseudomallei isolates with decreased antibiotic susceptibility compared to K96243.

| Genotype or phenotype | B. pseudomallei isolates |
|-----------------------|-------------------------|
|                       | DR10212A | DR30013A | DR80110A | DR90049A | DR50054A | DR50054E | DR90031A | DR90031E |
| **Antibiotic susceptibility** |
| CAZ                   | R         | R         | I         | S         | S         | R         | S         | S         |
| MEM                   | LS        | S         | R         | S         | LS        | S         | S         | S         |
| AMC                   | R         | S         | S         | S         | S         | S         | S         | S         |
| SXT                   | R         | S         | S         | S         | S         | S         | S         | S         |
| MLST                  | ST10      | ST10      | ST531     | ST207     | ST207     | ST207     | ST207     | ST207     |
| **β-lactamases**      |           |           |           |           |           |           |           |           |
| penA                  | P167S     | P167S     | WT        | WT        | I139M     | I139M     | WT        | WT        |
| *oxr-S9*              | WT        | WT        | WT        | WT        | T147A     | T147A     | WT        | WT        |
| *Up-stream of penA*   | WT        | -71A>G    | -71A>G    | WT        | -71A>G    | -71A>G    | WT        | WT        |
| **β-lactam target**   |           |           |           |           |           |           |           |           |
| PBSJ                  | WT        | WT        | WT        | WT        | WT        | WT        | WT        | WT        |
| **AmrAB-OpA efflux pump** |
| oprA                  | WT        | WT        | WT        | WT        | R104C     | WT        | WT        | WT        |
| *amrA*                | WT        | WT        | WT        | WT        | R116Q     | R116Q     | WT        | WT        |
| *amrB*                | WT        | WT        | WT        | WT        | WT        | WT        | WT        | WT        |
| *amrR*                | WT        | WT        | WT        | WT        | WT        | WT        | WT        | WT        |
| **BpeAB-OpR efflux pump** |
| *oprB*                | WT        | WT        | WT        | WT        | A501S     | A501S     | WT        | WT        |
| *bpeA*                | WT        | WT        | WT        | WT        | P390S     | P390S     | WT        | WT        |
| *bpeB*                | WT        | WT        | WT        | WT        | WT        | WT        | WT        | WT        |
| *bpeR*                | WT        | WT        | WT        | WT        | WT        | WT        | WT        | WT        |
| **BpeF-OpC efflux pump** |
| *oprC*                | WT        | WT        | WT        | WT        | V294A     | V294A     | WT        | WT        |
| *bpeE*                | WT        | WT        | WT        | WT        | V294A     | V294A     | WT        | WT        |
| *bpeT*                | WT        | WT        | WT        | WT        | W1546s    | W1546s    | WT        | WT        |
| *bpeS*                | WT        | WT        | WT        | WT        | V294A     | V294A     | WT        | WT        |
| **Outer membrane porin omp38** |
| WT                    | WT        | WT        | WT        | WT        | R143P     | R143P     | WT        | WT        |
| **Tetra-hydrofolate biosynthetic pathway** |
| *folA*                | WT        | WT        | WT        | WT        | V78A      | V78A      | WT        | WT        |
| *folI*                | WT        | WT        | WT        | WT        | V78A      | V78A      | WT        | WT        |

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A, primary isolate; E, relapse isolate; MLST, Multilocus sequence type; WT, wild-type; fs, frameshift mutation; del, deletion. The mutations coloured in blue were also found in antibiotic susceptible *B. pseudomallei* isolates. Those coloured in green were known CAZ-R variants [20,22]. Novel mutations found in this study were coloured in red.
Bacterial positive growth in clinical specimens (N=1,317)

Biochemical identification of *B. pseudomallei*

Antibiotic susceptibility testing in the hospitals

(i) Disk diffusion
- Hospital A
- Hospital B
- Hospital E
- Hospital H

(ii) Disk diffusion and E-test
- Hospital C
- Hospital F
- Hospital G

(iii) Disk diffusion, broth microdilution and BD Phoenix™
- Hospital D

(iv) Vitek®2
- Hospital I

Latex agglutination

MALDI-TOF MS

Disk diffusion testing for clinical *B. pseudomallei* isolates that were either not performed or reported as intermediate resistant or resistant by hospitals.
- (N=109, CAZ)
- (N=774, MEM)
- (N=253, IPM)
- (N=833, AMC)

Validation by broth microdilution
- (N=11, CAZ)
- (N=13, MEM)
- (N=6, IPM)
- (N=12, AMC)
- (N=10, SXT)
