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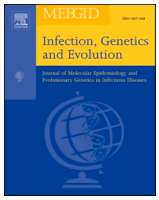

Whole-genome sequencing analysis of *Burkholderia pseudomallei* comparing drug-resistant and pan-susceptible isolates reveals novel biomarkers for drug resistance

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ABSTRACT

Melioidosis, caused by the gram-negative bacterium *Burkholderia pseudomallei* (Bp), poses a significant health threat due to its potential for drug resistance, which can severely limit available treatment options. To investigate this, we conducted a comparative genomic analysis of 38 drug-resistant (DR) and 300 drug-susceptible (DS) Bp isolates to identify genetic markers associated with antimicrobial resistance. Our study identified seven significant single-nucleotide polymorphisms (SNPs) linked to drug resistance: two with ceftazidime (CAZ), and five with meropenem (MEM). Pathway analysis revealed that AMC resistance was associated with alterations in fatty-acid metabolism, whereas CAZ resistance was associated with changes in membrane protein pathways. These findings highlighted how Bp develops resistance to key antibiotics through various mechanisms. In addition, we discovered 21 novel genetic variants in known drug-resistance genes, including 15 SNPs and six short insertions or deletions (indels). These previously unreported variants could contribute to resistance, highlighting the genetic diversity and adaptability to antimicrobial pressures of Bp. These findings deepen our understanding of Bp drug resistance and offer valuable insights into genetic markers with the potential to enhance diagnostic precision. By enriching the resistance database, this work provides prospective tools for early resistance prediction, facilitating prompt and effective treatment strategies. Furthermore, it emphasizes the critical role of genetic investigations in addressing the challenge of antibiotic resistance in melioidosis.

1. Introduction

Melioidosis is a potentially lethal infectious disease that is primarily prevalent in tropical and subtropical locations, especially in northern Australia and Southeast Asia. It is caused by the Gram-negative, motile, facultatively anaerobic bacterium *Burkholderia pseudomallei* (Bp) (Palasatien et al., 2008; Wang-ngarm et al., 2014). This environmental pathogen thrives in wet soils and stagnant water (Wiersinga et al., 2018), making it a significant risk factor for populations engaged in agriculture and activities involving soil and water contact (Leelarasamee et al., 1997). The bacteria can infect humans through ingestion, inhalation, or direct contact with infected soil or water (Dance, 1991; Palasatien et al., 2008; Wang-ngarm et al., 2014). Clinically, Bp infections manifest in a wide range of forms, from superficial wound infections to severe systemic conditions such as septicemia and pneumonia, which carry high fatality rates if left untreated. In Thailand, more than 5000 cases of melioidosis are reported annually (Wiersinga et al., 2018). As of 2016, melioidosis had the highest fatality rate of any infectious disease

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in the country—reaching up to 40 %—surpassing leptospirosis, dengue, and malaria, according to the Thai Bureau of Epidemiology (Melioidosis - Information, 2025; Wiersinga et al., 2018). The United States Centers for Disease Control and Prevention (CDC) has classified this pathogen as a potential high-priority biological weapon (Wang-ngarm et al., 2014).

A major challenge in managing melioidosis is the intrinsic antimicrobial resistance of Bp. The bacterium exhibits resistance to a broad range of antibiotics, including aminoglycosides, macrolides, and first-line penicillin (Fen et al., 2021; Khosravi et al., 2014; Wiersinga et al., 2018), due to its unique structural features and ability to produce multiple resistance mechanisms, such as efflux pumps and enzymatic degradation of drugs (Hayden et al., 2012; Price et al., 2013). The medications ceftazidime (CAZ), meropenem (MEM), imipenem (IPM), trimethoprim-sulfamethoxazole (SXT, also known as co-trimoxazole), and amoxicillin-clavulanic acid (AMC) are among the few treatment options available for patients in Thailand (Fen et al., 2021; Khosravi et al., 2014; Wiersinga et al., 2018). At present, there is a low frequency of Bp resistance to these drugs. For instance, resistance to CAZ in Thailand is estimated at only 0.1 to 1.5 %, while in Malaysia, it ranges from 0.6 to 2.4 % (Fen et al., 2021; Wiersinga et al., 2018). However, the global trend of increasing bacterial drug resistance is a growing concern (Hall et al., 2023; Madden et al., 2021; Sarovich et al., 2018). Given the limited treatment options, resistance in Bp can result in treatment failure (Sarovich et al., 2018). The scarcity of drug-resistance data, particularly in endemic regions, poses challenges for effective disease control and underscores the urgent need to prioritise melioidosis within global health policy. Monitoring the emergence of drug resistance in Bp is critically important to strengthen surveillance efforts, drive research into innovative diagnostic tools, and develop novel therapeutic strategies.

In recent years, whole-genome sequencing (WGS) has emerged as a transformative tool for studying Bp. WGS enables comprehensive genomic analysis, providing valuable insights into the genetic diversity, pathogenicity, and antimicrobial resistance mechanisms of this bacterium (Chapple et al., 2016; Fen et al., 2021; Sarovich et al., 2017). By delivering a complete map of the bacterial genome, WGS facilitates the identification of genes associated with drug resistance, virulence factors, and potential vaccine targets. Despite its potential, there have been few studies focusing on the genetic analysis of Bp in relation to drug susceptibility (Chen et al., 2025; Madden et al., 2021; Price et al., 2013), largely due to the limited availability of drug-resistant (DR) Bp strains. Few investigations have identified single-nucleotide polymorphisms (SNPs) linked to drug resistance. For example, variants in the beta-lactamase gene *penA* have been associated with resistance to AMC and CAZ. Similarly, SNPs in genes linked to resistance-nodulation-division (RND) multidrug efflux pumps (e.g., *AmrAB-OprA*, *BpeAB-OprB*, and *BpeEF-OprC*) have been implicated in resistance to MEM and SXT (Fen et al., 2021; Podnecky et al., 2017; Sarovich et al., 2018). These findings are based on small DR cohorts, leaving the catalogue of resistance markers incomplete. Comparative genomic analyses of DR versus drug-susceptible (DS) isolates remain rare—Madden et al. (2021) is a notable exception—but even such studies have not systematically assessed the predictive value of detected SNPs. Instead, most WGS work has focused on outbreak investigation and transmission dynamics (Aziz et al., 2020; Trevino et al., 2021). Consequently, the variants identified so far offer only limited power to predict resistance phenotypes. This study aimed to perform comparative genomics to analyse the genomes of DR and DS Bp isolates to identify novel biomarkers for predicting drug resistance. Potential biomarkers include SNPs and short insertions or deletions (indels), which could guide prescription decisions to minimise the development of drug resistance in melioidosis patients. Additionally, identifying significant genes encompassing these SNPs may provide mechanistic insights into drug resistance, further advancing therapeutic strategies.

2. Materials and methods

2.1. Study population and drug susceptibility test

In this study, we systematically retrieved FASTQ files from publicly accessible databases. The inclusion criteria were as follows: (1) sequencing data generated using the Illumina platform to ensure consistency in sequencing technology, and (2) the availability of drug susceptibility test results for each isolate sequenced. Specifically, each isolate had to exhibit resistance to at least one of the following antimicrobial agents—amoxicillin-clavulanate (AMC), ceftazidime (CAZ), meropenem (MEM), or co-trimoxazole (SXT)—or be confirmed as susceptible to all four drugs. Based on these criteria, we identified and included 38 FASTQ files corresponding to drug-resistant isolates. These datasets were derived from Bp strains collected in six provinces of Northeast Thailand ($n = 16$), as well as from publicly accessible databases containing Australian strains ($n = 22$) and one Thai strain (Supplementary Table 1). Additionally, a control group of pan-susceptible isolates consisted of 300 FASTQ files (Supplementary Table 2), which included sequences from Bp strains from our biobank covering nine provinces of Northeast Thailand ($n = 284$), Australian strains ($n = 14$), and Malaysian strains (n = 2). We included samples from BioProject No. PRJNA1051349, PRJNA285704, PRJNA300580, PRJNA272882, PRJNA412120, PRJNA393909, PRJNA274367, PRJNA343065, PRJNA285705. The MIC breakpoints for susceptibility, intermediate resistance, and resistance were determined according to the CLSI M100-S17 guidelines for Bp: AMC (≤8/4, 16/8, ≥32/16 μg/mL), CAZ (≤8, 16, ≥32 μg/mL), and SXT (≤2/38, NA, ≥4/76 μg/mL). For MEM, CLSI guidelines for Bp are lacking; therefore resistance was defined as ≥3 μg/mL based on prior studies and EUCAST recommendations (Crowe et al., 2014; Maloney et al., 2017). This rigorous selection process allowed us to create a robust dataset for downstream analyses. The study protocol was approved by the Khon Kaen University Ethics Committee for Human Research (approval no. HE641201).

2.2. Whole-genome sequencing and data processing

The quality of whole-genome sequencing (WGS) data was assessed using FastQC (version 0.11.9) (Babraham Bioinformatics, 2025). Low-quality regions were trimmed using Trimmomatic software (v0.38) (Bolger et al., 2014). High-quality reads were mapped to the *Burkholderia pseudomallei* K96243 reference genome (chromosome 1: NC_006350.1; chromosome 2: NC_006351.1) (Holden et al., 2004) using BWA-MEM software (v0.7.12) (Li, 2014). The resulting BAM files were sorted and indexed with SAMtools (v1.15.1) (Li et al., 2009). Reads were realigned using GATK (version 3.6.0) (McKenna et al., 2010), and variants were called with GATK and BCFtools (v1.2) (Danecek et al., 2021), focusing on single-nucleotide polymorphisms (SNPs) and short insertions/deletions (indels) of length less than 50 bp (Jugas and Vitkova, 2024). Variants were filtered using stringent thresholds—mapping quality ≥50 (≈99.999 % alignment confidence), base quality ≥20 (≤1 % sequencing-error rate), and read depth ≥ 10. These cut-offs were chosen after inspecting variant-count distributions in our Bp dataset and are intended to remove low-confidence calls that are likely sequencing artefacts. Variants identified by both tools were analysed further. SAMtools mpile-up files and variant-calling files (VCFs) were used to generate a combined isolate file of nucleotide frequencies, allowing for SNP position analysis. The multiple sequence alignment of these combined variants was used to construct the phylogenetic tree. Maximum-likelihood phylogenetic trees were constructed with IQ-TREE version 1.6.9, using 1000 bootstrap replicates and the GTR + G + ASC model (General Time-Reversible with discrete Gamma distribution and ascertainment bias correction) (Nguyen et al., 2015). The final circular phylogenetic tree, based on the complete dataset, was visualised using iTOL (Letunic and Bork, 2021).

2.3. Drug-resistant variant analysis

Variants identified as above were used to compare DS and DR Bp isolates for each drug. In our study, DS refers to isolates susceptible to AMC, CAZ, MEM, and SXT. The VCF files from each drug were combined to identify variants common to all isolates within the group. These common variants for each drug were compared against the DS group to identify unique variants. The unique variants were then cross-referenced with the union of variants from the pan-susceptible group to identify drug resistance-specific variants (Supplementary Fig. S1). Additionally, another approach was used to identify variants associated with drug resistance. The VCF files from the DR and DS groups were combined, and a two-by-two contingency table was constructed for each genomic position. The Fisher's exact test was applied to calculate p values, with significant positions selected for further analysis. All processes in this section were performed using in-house Python scripts. High-confidence variants were considered those with a Fisher exact p value <0.05 and an absence from any DS isolate. Additionally, known variants and genes were also included (Supplementary Table 7).

2.4. Gene annotation

All significant genomic positions were annotated to their respective genes using the genome of *Burkholderia pseudomallei* strain K96243 as a reference (chromosome 1: NC_006350.1; chromosome 2: NC_006351.1) (Holden et al., 2004). Specifically, a general feature format (GFF3) file of the reference genome served as the primary template for identifying genes associated with each variant position. An in-house Python script was employed to perform the annotation. In addition to this approach, variant annotation was independently conducted using the SnpEff tool (Cingolani et al., 2012). The results from both methods were cross-referenced to identify overlapping annotations, ensuring accuracy and consistency. The intersecting results were compiled into a final annotated gene file for downstream analysis.

2.5. Network and gene-enrichment analysis

The genes corresponding to each DR variant were subjected to network analysis. The included genes were analysed for protein-protein interactions using the STRING database (https://string-db.org). Subsequently, the network was visualised and refined using Cytoscape software (version 3.10.3) (Shannon et al., 2003). To identify highly interactive genes, the cytoHubba plugin (version 0.1) within Cytoscape was utilised. The network of 50 highly interacting genes of maximum clique centrality (MCC) was used as a starting point for network analysis. Gene-enrichment analysis was conducted using data from STRING, based on Gene Ontology (GO) classifications. The GO annotations were categorised into three domains: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC). Visualisation of the enriched categories was performed using the online platform SRplot (https://www.bioinformatics.com.cn/en) (Tang et al., 2023), which facilitated the generation of bubble graphs for the data representation.

2.6. Hub gene identification

For further analysis, the 50 highly interactive genes were identified using six analytical techniques, incorporating three locally ranked parameters—degree, maximum neighbourhood component (MNC), and maximum clique centrality (MCC)—and three globally ranked parameters: closeness centrality, betweenness, and the stress method (Bristy et al., 2023). The resulting gene sets were further analysed and visualised using jvenn, a web-based tool for Venn diagram analysis (Bardou et al., 2014). The intersection of these sets was identified and included genes designated as hub genes. Those significant genes were further reviewed for associations with drug resistance. The variants within those genes, along with their associations with drug resistance, were further evaluated using adjusted p-values calculated by the Benjamini-Hochberg FDR method.

3. Results

3.1. Distribution and drug-resistance patterns of Bp isolates

Of the 338 Bp isolates examined in this study, 300 (88.75 %) were DS isolates and 38 (11.25 %) were DR isolates. The DS isolates originated from hospitals in nine provinces of Northeast Thailand ($n = 284$; 94.67 %), Australia ($n = 14$; 4.67 %), and Malaysia (n = 2; 0.66 %) (Supplementary Table 1). The DR isolates were collected between 2004 and 2021 from hospitals in six provinces of Northeast Thailand ($n = 17$; 44.74 %) and Australia ($n = 21$; 55.26 %) (Fig. 1A, Supplementary Table 2). Among the DR isolates, resistance to meropenem (MEMr) was the most common (n = 21; 55.26 %), followed by resistance to co-trimoxazole (SXTr; n = 14; 36.84 %), amoxicillin-clavulanic acid (AMCr; $n = 9$; 23.68 %), and ceftazidime (CAZr; $n = 7$; 18.42 %). Notably, twelve isolates demonstrated resistance to more than one drug. The phylogenetic analysis revealed a broadly dispersed distribution of isolates, with no clustering by country of origin or overall drug-susceptibility profile. Nevertheless, a distinct clonal lineage of CAZ-, MEM-, and SXT-resistant isolates was detected among the Australian strains (Fig. 1B).

3.2. Known SNPs associated with drug-resistant Bp and novel variants of known resistance genes

To identify genetic markers associated with drug resistance for each drug, a comparative genomic analysis was conducted. The initial approach compared genetic variants commonly found in DR strains with those prevalent in DS strains. However, no unique variants were exclusively present in DR strains and absent in all DS strains (Supplementary Fig. S1). Subsequently, an alternative approach was adopted, considering the possibility of genetic markers appearing in multiple positions within a gene or being present only in specific DR strains. To enhance sensitivity in detecting potential DR variants, variants meeting stringent criteria—p-value <0.05 and complete absence from DS isolates—were selected. A total of 1317, 1245, 310, and 1070 raw unique variants were associated with resistance to AMC, CAZ, MEM, and SXT, respectively (Fig. 1C, Supplementary Tables 3–6). These identified variants provide a valuable resource for further gene-level and downstream analyses.

We identified previously reported SNPs associated with known drug-resistance genes in a well-established database of Bp (Supplementary Table 7). These included SNPs associated with resistance to AMC (three SNPs; 1 in *BPSL0731*; *bpeS*, and 2 in *BPSS0946*; *penA*), CAZ (four SNPs; 1 in *BPSL0731*; *bpeS*, and 3 in *BPSS0946*; *penA*), MEM (one SNP in *BPSL1805*; *amrR*), and SXT (seven SNPs; 1 in *BPSL0731* (*bpeS*), 2 in *BPSL0903* (*dut*), 1 in *BPSL1805* (*amrR*), 1 in *BPSL3288* (*metF*), 1 in *BPSS0290* (*bpeT*), and 1 in *BPSS0946* (*penA*)). Additionally, novel variants in known resistance genes were identified, including variants associated with resistance to AMC (three SNPs), CAZ (four SNPs and one short indel), MEM (three SNPs), and SXT (six SNPs and five short indels).

For those novel variants from known genes, there were three SNPs putatively linked to AMC resistance across *BPSL0815* (G944946A), *BPSL1803* (T2149037C), and *BPSL3288* (G3905647A). There were four SNPs and one short indel for CAZ resistance, *BPSL0816* (G947877C), *BPSL1802* (G2147372A), *BPSS0290* (T388928G), and *BPSS0946* (C1248733T), and *BPSL1805* (2152343ΔCGAAGA → C). In Bp isolates with MEMr, there were two genes with three SNPs, *BPSL0814* (A943089G and T944044C) and *BPSL1803* (G2148454A). There were eight genes with six SNPs and five short indels for SXTr: *BPSL0731* (A845163G), *BPSL0815* (A944297G), *BPSL1802* (C2147681T), *BPSL1803* (G2149344A), *BPSL3288* (T3905254G), *BPSS1219* (G1648250A), *BPSL1805* (2152757ΔAGCTCGACGCGTCGCTCGACG →

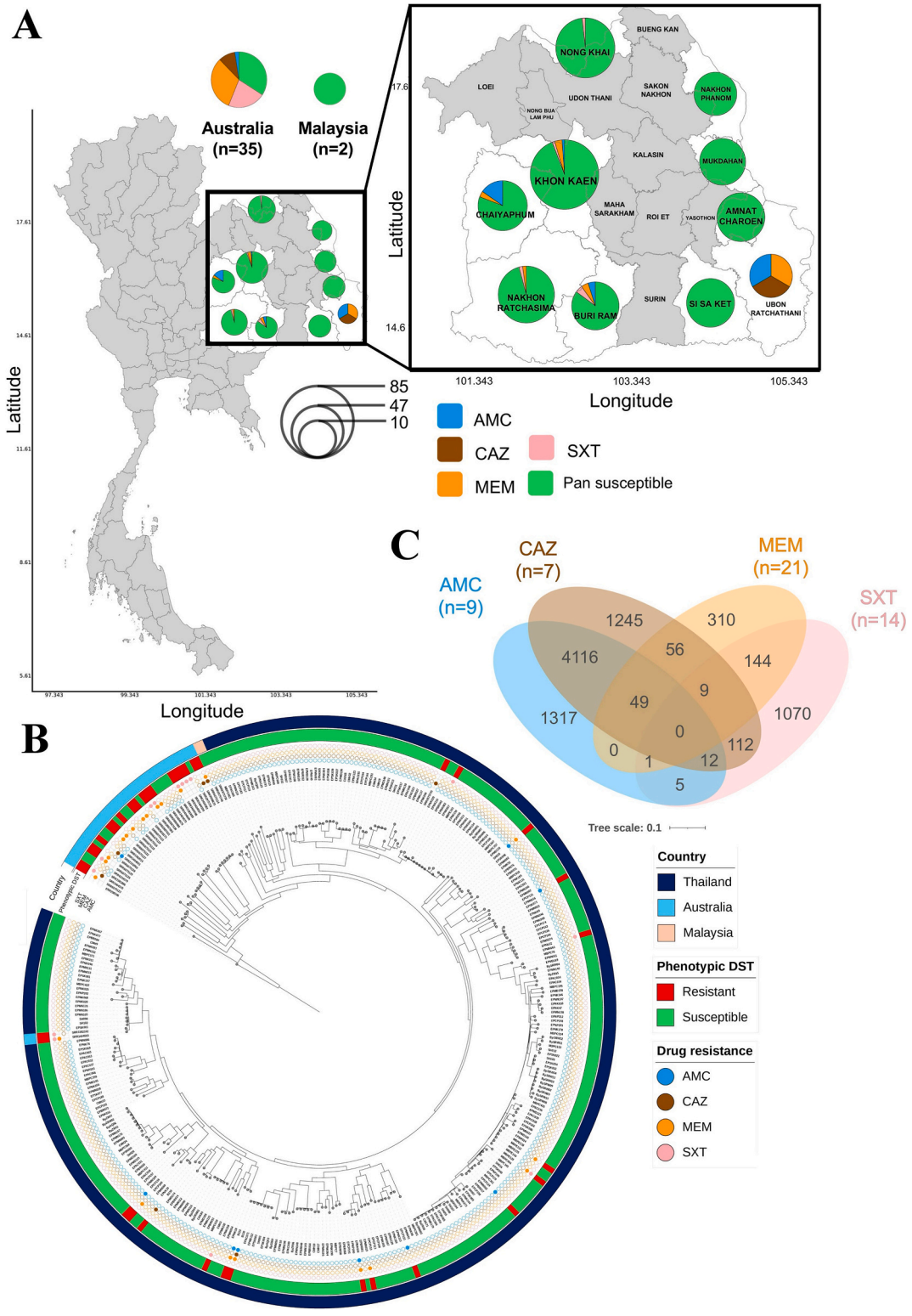

Fig. 1. The distribution of our data. (A) A geographical map of our isolates illustrated their distribution and corresponding drug resistance profiles. (B) The phylogenetic tree illustrated the distribution of isolates included in this study. The outer strip line indicated the country of origin, the middle strip line represented the results of phenotypic drug susceptibility testing, and the inner binary dot denoted the presence or absence of drug resistance. (C) A Venn diagram illustrating the number of variants identified that were associated with drug resistance. The analysis revealed unique variants specific for each drug, with no variants shared across all drugs. AMC: Amoxicillin-clavulanic acid, CAZ: Ceftazidime, MEM: Meropenem, and SXT: Co-trimoxazole.

AGCTCGACG), *BPSL3288* (3904961ΔGT → G), and *BPSS0039* (39343ΔCCGCGCGGCGCGCGGCGC → CCGCGCGGCGC, 39349GG_insGGGGCGCGCCG and 39356G_insGCGGCGCGCC) (Supplementary Tables 3–6).

3.3. Gene-network analysis identified the possible pathways associated with drug resistance

The raw unique variants identified in the previous analysis step were subjected to network analysis to identify potential candidate genes associated with drug resistance in Bp. Genes encompassing each variant were included in the analysis, resulting in the identification of 495 genes for AMC, 518 genes for CAZ, 146 genes for MEM, and 435 genes for SXT. The top 50 highly interacting genes, determined using the maximum clique centrality (MCC) parameter, were visualised (Fig. 2). The interaction network revealed 650 edges for AMC, 544 edges for CAZ, 139 edges for MEM, and 412 edges for SXT. These findings are summarised in Fig. 2, showcasing the interaction networks and the key genes involved.

In the analysis of genes associated with unique variants, several biological processes were notably enriched. Among these, the fatty-acid biosynthetic process showed the highest significance and enrichment for both AMC and CAZ (AMC: $p = 0.0012$, enrichment = 0.59; CAZ: $p = 0.0225$, enrichment = 0.53). This was followed by the monocarboxylic-acid biosynthetic process (AMC: $p = 0.005$, enrichment = 0.49; CAZ: $p = 0.0266$, enrichment = 0.46) and the fatty-acid metabolic process (AMC: $p = 0.0093$, enrichment = 0.41; CAZ: p = 0.0266, enrichment = 0.39). Genes within these categories were exclusively observed in AMC and CAZ. For molecular functions, phosphopantetheine binding emerged as the most significant across AMC, CAZ, and SXT (AMC: $p = 3.98 \times 10^{-7}$, enrichment = 0.92; CAZ: $p = 4.15 \times 10^{-6}$, enrichment = 0.87; SXT: $p = 4.50 \times 10^{-5}$, enrichment = 0.88). This was followed by 3-oxoacyl-[acyl-carrier-protein] synthase activity and amide binding. Notably, CAZ alone exhibited significant enrichment in vitamin binding and zinc ion binding. Furthermore, two molecular function pathways, 3-oxoacyl-[acyl-carrier-protein] synthase activity and phosphopantetheine binding, were found to be common across all drugs. Regarding cellular components, these were exclusively identified in CAZ and SXT. For CAZ, the membrane showed the highest significance ($p = 0.0006$, enrichment = 0.12), followed by the integral and intrinsic components of the membrane (p = 0.001, enrichment = 0.14). For SXT, the cell periphery was most significant ($p = 0.0017$, enrichment = 0.16), followed by the membrane ($p = 0.0027$, enrichment = 0.11) and cellular anatomical entity (p = 0.0027, enrichment = 0.04). These findings are summarised in Fig. 3 and Supplementary Table 8.

3.4. Identification of possible genes associated with resistance against each drug

Following network analysis, the top 50 most highly interacting genes were subjected to candidate gene analysis using six parameters based on gene interaction criteria. Genes meeting all six criteria were identified as hub genes (Supplementary Figs. S2–S5). Twelve hub genes were identified for AMC, six of which were unique to this drug. CAZ yielded 20 hub genes, including ten unique ones. MEM displayed the largest set—24 hub genes, 19 of which were unique—whereas SXT had 18 hub genes, nine unique to SXT (Fig. 4). Notably, *BPSS1008* consistently achieved the highest score across all drugs, aligning with findings from the analysis of genes associated with all SNPs (Table 1, Supplementary Table 9).

Unique genes associated with resistance from our review are listed in Supplementary Table 10. For AMC, two genes were identified, each harbouring a single SNP: *BPSL3218* (*rpsL*), with the SNPs C3813784T, and *BPSS1940*, with the SNP G2627176A. Similarly, for CAZ, two genes

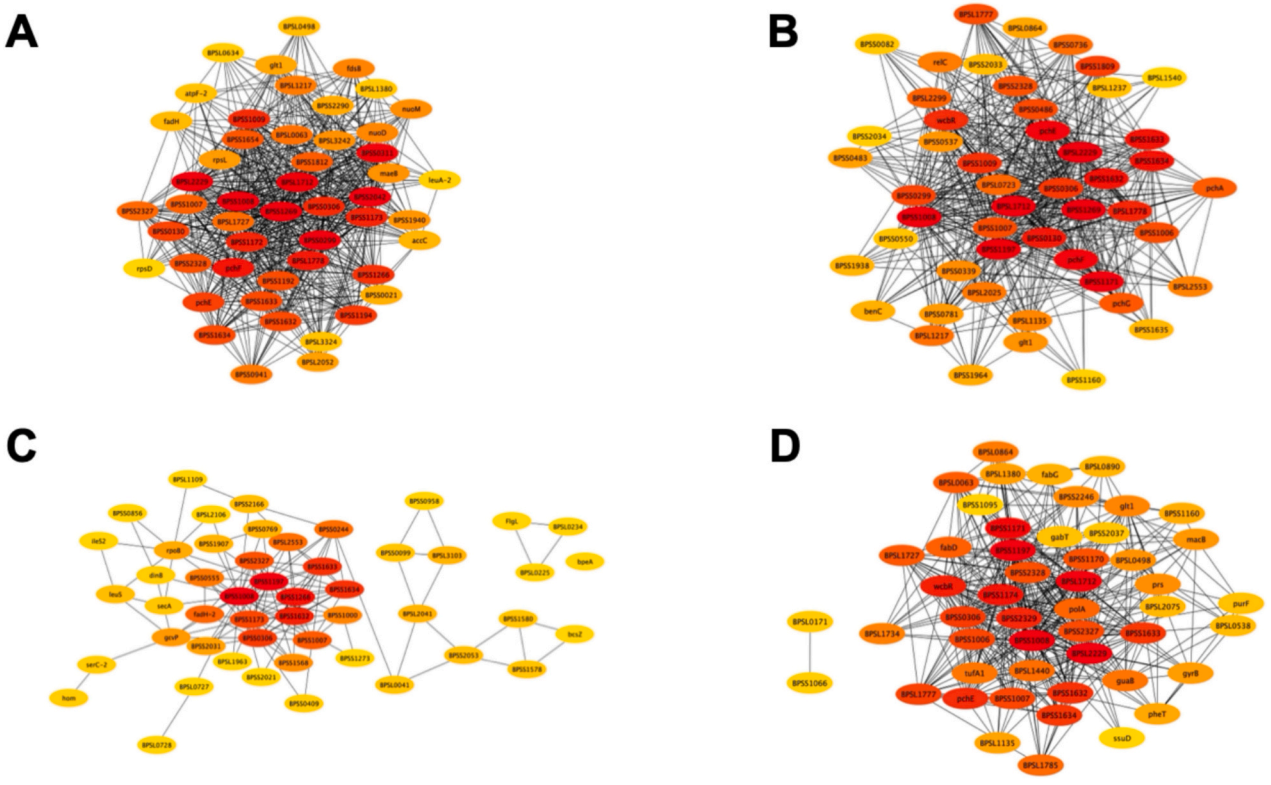

Fig. 2. Network analysis was conducted on the 50 most highly interacting genes based on maximum clique centrality (MCC). A, B, C, and D) Networks corresponding to genes associated with AMC, CAZ, MEM, and SXT, respectively. Node colours indicate interaction intensity, ranging from high (red) to low (yellow), providing a visual representation of the centrality and importance of each gene within the network. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

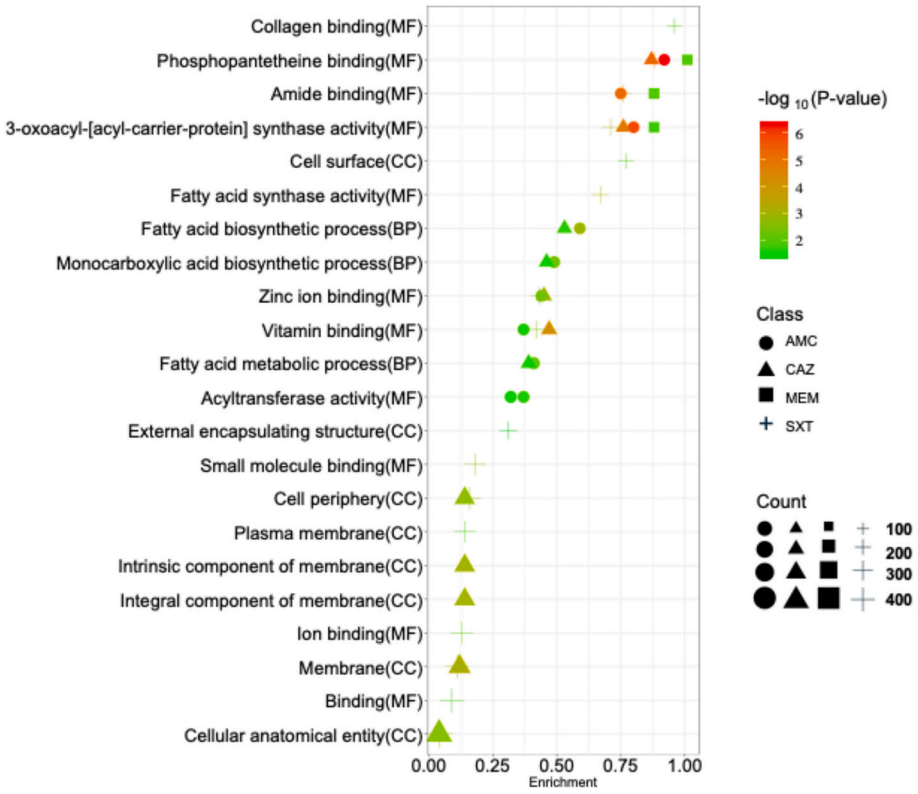

Fig. 3. Gene enrichment analysis using ontologies. Gene enrichment bubble graph of genes from unique SNPs. BP; biological process, MF; molecular function, CC; cellular component, AMC; amoxicillin-clavulanic acid, CAZ; ceftazidime, MEM; meropenem and SXT; co-trimoxazole. The x-axis indicates enrichment values.

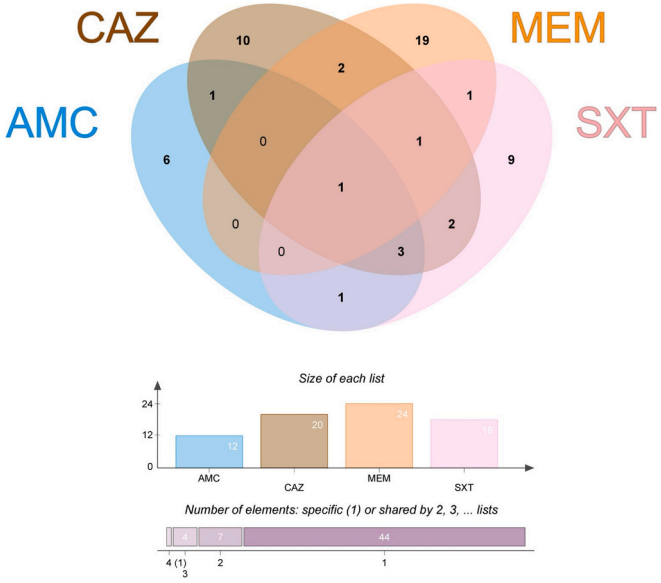

Fig. 4. Venn diagram representing the hub genes across all drugs. AMC had 12 hub genes, including six unique for this drug. CAZ exhibited 20 hub genes, with 10 being unique. MEM had the highest number, with 24 hub genes, 19 of which were unique. SXT had 18 hub genes, including nine unique for this drug.

were detected, each with one SNP: *BPSL2025*, with G2418582A, and *BPSS0588* (*pchG*), with C807365T. For MEM, four genes and five SNPs were identified: *BPSL0041* (G41420A), *BPSL3016* (*secA*, G3593982A), *BPSL3221* (*rpoB*, G3823367A and C3823415T), and *BPSS0244* (T332714C). Finally, for SXT, a single gene, *BPSS0624* (*macB*), was associated with resistance through the SNP T852904A (Table 1, Supplementary Table 11).

Table 1
List of hub genes found for each drug.

Drug	Common genes	Unique genes
AMC	*BPSL0498, BPSL1712, BPSL2229,* ***BPSL3159 (glt1)****, BPSS1008, BPSS1269*	*BPSL2959 (maeB), BPSL3188 (rpsD),* ***BPSL3218 (rpsL)****, BPSL3242, BPSS1654,* ***BPSS1940****
CAZ	*BPSL1135, BPSL1712, BPSL2229,* ***BPSL2553****, BPSL2789 (wcbR),* ***BPSL3159 (glt1)****, BPSS0306, BPSS1008, BPSS1197, BPSS1269*	*BPSL0723,* ***BPSL2025****, BPSL2299, BPSL3225(relC), BPSS0339, BPSS0486, BPSS0550,* ***BPSS0588 (pchG)****, BPSS0736, BPSS1964* ***BPSL0041****, BPSL2041, BPSL2306 (dinB), BPSL2938 (leuS),* ***BPSL3016 (secA)****,* ***BPSL3221 (rpoB)****,*
MEM	***BPSL2553****, BPSS0306, BPSS1008, BPSS1197, BPSS1632*	*BPSL3362 (gcvP),* ***BPSS0244****, BPSS0409, BPSS1000, BPSS1007, BPSS1173, BPSS1266, BPSS1633, BPSS1634, BPSS1898 (fadH-2), BPSS2053, BPSS2166, BPSS2327*
SXT	*BPSL0498, BPSL1135, BPSL1712, BPSL2229, BPSL2789 (wcbR),* ***BPSL3159 (glt1)****, BPSS1008, BPSS1197, BPSS1632*	*BPSL1440, BPSL1940 (pheT), BPSL2129 (guaB), BPSL3228 (tufA1),* ***BPSS0624 (macB)****, BPSS1095, BPSS1171, BPSS1770 (polA), BPSS2246*

* and bold letter indicates genes previously known to be associated with drug resistance. "Unique genes" are hub genes detected only in isolates resistant to a single drug, whereas "common genes" are hub genes shared by two or more drug-resistant groups.

4. Discussion

Drug-resistant melioidosis is not common, and identifying genetic markers associated with DR Bp remains challenging. For this investigation, we utilised DR strains obtained from Thailand and public databases. The study focused on isolates resistant to four antibiotics: AMC,

CAZ, MEM, and SXT. Among these, MEM-resistant isolates were the most prevalent, followed by SXT, AMC, and CAZ. We observed that MEM-resistant and AMC-resistant isolates constituted the largest share of drug-resistant Bp in northeastern Thailand (0.027 %), followed by resistance to SXT and CAZ. These frequencies differ markedly from earlier reports, which documented MEM- and AMC-resistance rates of 2 % and 5 %, respectively (Panya et al., 2016; Paveenkittiporn et al., 2009). Because the present analysis was limited to strains available in our biobank and sequencing dataset, the figures presented here may not fully represent the true regional epidemiology of antimicrobial resistance.

DR and DS Bp isolates were compared to identify candidate resistance markers. We first assumed that variants present in every DR isolate but absent from all DS isolates would constitute robust markers. Although several DR-specific variants emerged, none were shared by the entire DR set—an observation consistent with Madden et al. (2021). To improve sensitivity, we next compared the union of variants from all DR isolates with the variant set from DS isolates. Variant positions significantly enriched in the DR group were identified using Fisher's exact test ($p < 0.05$) and subsequently subjected to network analysis and hub-gene prioritisation. The genes associated with unique variants were then utilised as inputs for network analysis to improve specificity. Gene Ontology (GO) pathways were employed to analyse gene enrichment, revealing that the identified biological processes were predominantly related to the fatty-acid biosynthetic process (GO:0004312), followed by the monocarboxylic-acid biosynthetic process (GO:0072330) and the fatty-acid metabolic process (GO:0004312).

The type II fatty-acid biosynthetic pathway supports cell-membrane formation and can promote drug resistance by lowering membrane permeability (Radka and Rock, 2022). Up-regulation of this pathway has been linked to multidrug resistance in *Helicobacter pylori* and to fluoroquinolone resistance in *Edwardsiella tarda* (Su et al., 2021; Xue et al., 2024). Enzymes within the pathway are therefore regarded as promising antimicrobial targets (Radka and Rock, 2022). In our dataset, enrichment of fatty-acid biosynthesis genes was confined to AMC- and CAZ-resistant isolates, whereas the two other biological processes highlighted by the analysis have not yet been implicated in bacterial drug resistance. For molecular functions, we identified phosphopantetheine binding (GO:0031177), 3-oxoacyl-[acyl-carrier-protein] synthase activity (GO:0004315), and amide binding (GO:0033218). Notably, 3-oxoacyl-[acyl-carrier-protein] synthase activity, which is part of the fatty-acid biosynthetic process, was detected for all four drugs, suggesting a potential link to drug resistance (Su et al., 2021; Xue et al., 2024). In terms of cellular components, enriched pathways were observed only in CAZ- and SXT-resistant strains. For CAZ, most enriched components were associated with membranes, including the membrane (GO:0016020), integral components of the membrane (GO:0016021), and intrinsic components of the membrane (GO:0031224). These membrane components are directly related to multidrug resistance by limiting the influx of antibiotics, such as beta-lactams, fluoroquinolones, and tetracyclines (Gauba and Rahman, 2023). For SXT, the identified cellular components included the cell periphery (GO:0071944), membrane (GO:0016020), and cellular anatomical entity (GO:0110165). Among these, only the membrane was associated with drug resistance.

The hub genes were identified using six parameters and the intersection of the top 50 highly interacting genes. Variants in these hub genes were then regarded as associated with drug resistance. These six parameters were chosen due to their high correlation, which provided reliable and stable results(Bristy et al., 2023; Chin et al., 2014). Our analysis revealed nine genes, and ten SNPs associated with drug resistance (Supplementary Tables 11). Among these, *BPSL3218* (*rpsL)* was unique for AMC. This gene encodes the 30S ribosomal protein S12, which has been linked to streptomycin resistance in *Mycobacterium tuberculosis*. Streptomycin binds to ribosomal protein S12, and variants in *rpsL* can affect binding affinity, leading to drug resistance (Khosravi et al., 2017). *BPSL2025*, a unique gene for CAZ, and *BPSS1940*, unique to AMC, encode proteins within the ATP-binding cassette (ABC) superfamily of ATPases. These proteins are known for their role in antibiotic resistance, primarily by reducing intracellular drug concentrations through active efflux (Lubelski et al., 2007). *BPSL3016* (*secA*), a unique gene for MEM, has also been implicated in carbapenem resistance. A study on *Acinetobacter baumannii* identified OXA-58 as a commonly acquired gene for carbapenem resistance, with its translocation linked to a SecA-dependent pathway. Inhibiting *secA* in combination with carbapenem was found to have a synergistic effect against carbapenem-resistant *A. baumannii* (Chiu et al., 2016). *BPSL3221* (*rpoB*), another unique gene for MEM, encodes the DNA-dependent RNA polymerase beta-subunit, a known target of rifampicin. Mutations in *rpoB* have been associated with rifampicin resistance in *M. tuberculosis* (Li et al., 2021). *BPSL2553*, found in both CAZ and MEM, and *BPSS0244*, unique to MEM, encode a TonB-dependent copper receptor, an outer membrane protein. A previous study demonstrated that deleting the corresponding receptor gene in *A. baumannii* significantly reduced biofilm formation. Additionally, the copper receptor-deficient mutant exhibited decreased adherence to human epithelial cells and reduced surface hydrophobicity (Abdollahi et al., 2018; Antunes et al., 2011). Phylogenetic analysis revealed clonal patterns among isolates resistant to CAZ, MEM, and SXT. Variants associated with CAZ resistance were present in two of seven isolates, and MEM resistance variants were detected in two of 21 isolates. Because these four isolates were not clonally related, clonal interference is unlikely to have biased our resistance-marker analysis. In addition, three variants—BPSL3218 (*rpsL*, AMC-specific), BPSS1940 (AMC-specific), and BPSS0624 (*macB*, SXT-specific)—were excluded because their adjusted *p*-values exceeded 0.05.

Known variants were identified by analysing all significant variant positions, revealing DR gene variants for each drug. However, some discordances in SNP associations were observed. For instance, *BPSL0731* (*bpeS*, C844449T), a SNP associated with SXT resistance, was detected in an AMC- and CAZ-resistant isolate (SRR2102060). Although this isolate exhibited phenotypic resistance to both AMC and CAZ, it did not show resistance to SXT. The *bpeS* gene is associated with the expression of the RND efflux pump via the production of the regulator protein BpeS, which regulates the BpeEF-OprC efflux pump (Rhodes et al., 2018). While RND efflux pumps are often linked to multidrug resistance, no evidence currently links them to beta-lactam resistance in Bp (Schweizer, 2012; Tribuddharat et al., 2003). However, in other bacteria, such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, efflux pumps have been reported to be associated with beta-lactam resistance (Dulanto Chiang and Dekker, 2024). In AMC-resistant isolates, we identified a SNP in *BPSS0946* (*penA*, A1248928G), which is linked to CAZ resistance. Similarly, in CAZ-resistant isolates, we observed another SNP in *BPSS0946* (*penA*, C1248427T), which is associated with AMC resistance. These SNPs are linked to beta-lactamase upregulation, which can confer resistance to CAZ due to high beta-lactamase activity (Bugrysheva et al., 2017). For AMC resistance, the A1248928G substitution causes an amino-acid change from aspartic acid to glycine, potentially altering the catalytic site kinetics of the enzyme, thereby reducing AMC susceptibility (Tribuddharat et al., 2003). In SXT-resistant isolates, we identified seven SNPs, five of which were directly associated with SXT resistance. Additionally, one SNP in *BPSL1805* (*amrR*, T2152703C) was linked to MEM resistance, while another SNP in *BPSS0946* (*penA*, G1248418A) was associated with CAZ resistance. The *BPSL1805* SNP (*amrR*, T2152703C) was identified in isolate SRR3404598, which exhibited phenotypic resistance to both MEM and SXT, likely explaining the presence of this SNP. Similarly, the *BPSS0946* SNP (*penA*, G1248418A) was detected in the SRR3404570 isolate, which was resistant to both SXT and CAZ (Supplementary Table 1).

Interestingly, we identified 22 novel variants in known DR genes. Of these, three SNPs were associated with AMC resistance. Two of these SNPs were found in genes encoding efflux pumps, while the third, *BPSL3288*, encodes methylenetetrahydrofolate reductase, which is

linked to SXT resistance. This gene plays a crucial role in the production of methyl tetrahydrofolate, an essential molecule that provides one-carbon units for methionine synthesis and is vital for the recycling and homeostasis of tetrahydrofolate (Matthews et al., 1998; Yu et al., 2025). Despite its role in folate metabolism, no reports have previously linked this gene to AMC resistance. For SXT, there were efflux pump genes, methylenetetrahydrofolate reductase, putative pteridine reductase, and penicillin-binding protein. We also observed aggregation of variants in known DR genes, which were BPSL3288 (*metF*) for 2 variants and BPSS0039 (*ptr1*) for three variants. BPSS0039 encoded putative pteridine reductase. This gene shares genetic similarities with *folM* gene in *Escherichia coli,* which has been primarily associated with co-trimoxazole resistance, and both names were usually used in the DR gene of Bp (Podnecky et al., 2017; Price et al., 2013). For SXT resistance, we also found a variant in *BPSS1219* (*PBP*), which encodes a penicillin-binding protein. The mechanism here is typically related to beta-lactam resistance through alterations in the target site—penicillin-binding proteins (Schweizer, 2012). Similarly, isolate SRR3404598, which was resistant to both MEM (a beta-lactam) and SXT, showed an SNP in *BPSS1219*, further supporting the association between this variant and resistance to both drugs. This position was also presented in MEM data, but it was not included due to a non-significant result (p value = 0.06542). Thus, this position should be excluded from novel variants from known genes of MEM.

The limitations of this study should be acknowledged. We conducted a comparative genomic analysis of drug-resistant Bp for four treatment drugs: AMC, CAZ, MEM, and SXT. Imipenem (IPM) was not included in our analysis because no resistance to this drug was observed in either our biobank or publicly available databases. Additionally, the number of isolates available for each antimicrobial agent depended on database content, resulting in unequal representation across drugs. Because drug-resistant Bp remains rare, the total number of resistant isolates was small, and smaller cohorts generally yield fewer detectable SNPs. To lessen this limitation, we validated candidate markers against a large panel of pan-susceptible isolates (n = 300). Nonetheless, a broader collection of drug-resistant genomes would further improve the accuracy and generalisability of our findings. Clonal isolates were included, but none overlapped within the drug-specific comparison sets, minimising clonal bias. Even so, confirming these variants in independent, non-clonal datasets is a priority for future work. Furthermore, the drug-resistance patterns analysed in this study may not fully represent the actual proportions of drug resistance in Bp populations, as our dataset consists of data aggregated from various studies. As such, the drug-resistance data in this study reflects the available data, rather than real-world prevalence.

In conclusion, we identified seven significant SNPs in resistance-associated genes along with their underlying mechanisms. Additionally, we reported 21 novel variants in known resistance genes. These SNPs have the potential to serve as reliable predictors of drug resistance. We also validated the known SNPs found in our isolates, reinforcing their robustness. Further investigation is needed to better understand the extent of drug resistance and the geographical distribution of resistant strains.

CRediT authorship contribution statement

Yothin Hinwan: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis. **Nut Nithimongkolchai:** Validation, Software, Formal analysis. **Kanwara Trisakul:** Writing – review & editing, Validation, Supervision, Formal analysis. **Benjawan Kaewseekhao:** Validation, Supervision, Formal analysis. **Lumyai Wonglakorn:** Supervision, Resources, Methodology, Data curation. **Ploenchan Chetchotisakd:** Writing – review & editing, Resources, Project administration, Data curation. **Sorujsiri Chareonsudjai:** Writing – review & editing, Supervision, Resources, Methodology. **Auttawit Sirichoat:** Validation, Supervision, Project administration, Methodology. **Arnone Nithichanon:** Writing – review & editing, Project administration, Methodology. **Jody Phelan:** Writing – review & editing, Supervision, Data curation. **Taane G. Clark:** Writing – review & editing, Supervision, Data curation. **Kiatichai Faksri:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Ethical approval

The study protocol was approved by the Khon Kaen University Ethics Committee for Human Research (approval no. HE641201).

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Declaration of competing interest

No potential conflict of interest was reported by the author(s).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2025.105779.

Data availability

Our whole-genome sequencing data (.fastq) have been deposited in GenBank under BioProject PRJNA1051349. Data retrieved from previous studies are available in BioProjects PRJNA1051349, PRJNA285704, PRJNA300580, PRJNA272882, PRJNA412120, PRJNA393909, PRJNA274367, PRJNA343065, PRJNA285705.

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