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Burkholderia cepacia Complex in King Chulalongkorn Memorial Hospital: Genomic Characterization and Antibiotic Susceptibility

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Abstract

The *Burkholderia cepacia* complex (Bcc) comprises gram-negative opportunistic pathogens. They cause infections in healthcare settings, particularly in individuals with weakened immune systems or lung conditions like cystic fibrosis (CF). Bcc bacteria often possess multiple antibiotic resistance genes on both chromosomes and plasmids. Although Thailand reports fewer Bcc infections than America and Australia, *Burkholderia cenocepacia* is a common species that causing infections. Reports indicate that Bcc bacteria can transfer genes, including those conferring antibiotic resistance, to other species within the genus, such as *B. pseudomallei*, which is prevalent in Thailand. This gene transfer raises concerns about the spread of antibiotic resistance. This study aims to characterize the genetic features of Bcc isolates obtained from patients at King Chulalongkorn Memorial Hospital in Thailand. We used *gyrB* gene sequencing to screen for *B. cenocepacia* and determined their susceptibility to ceftazidime (CAZ), meropenem (MEM), and trimethoprim-sulfamethoxazole (SXT) using the broth microdilution method. The results showed that all *B. cenocepacia* isolates in our sample were susceptible to all three antibiotics. Additionally, we performed whole genome sequencing of the *B. cenocepacia* SCBC75 and compared its sequence with a reference strain J2315. The analysis revealed genomic differences between these two strains. Notably, we identified numerous antibiotic resistance genes on the chromosomes of the *B. cenocepacia* isolate. These findings highlight the importance of genomic studies in identifying potential resistance mechanisms, even in isolates that appear susceptible to current treatments.

Keywords: *Burkholderia cepacia* complex, Antibiotic resistance, *gyrB* sequencing, Whole genome sequencing

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Introduction

Gram-negative bacteria in the *Burkholderia cepacia* complex (Bcc) are closely related, and while most are beneficial, some can have serious negative impacts on human health, especially in individuals with compromised immune systems, cystic fibrosis (CF), or chronic granulomatous disease (CGD). *Burkholderia cenocepacia* is the most clinically significant species in the Bcc group because it is associated with severe diseases, easily transmitted between patients, and resistant to several medications (Drevinek & Mahenthiralingam, 2010; Jin et al., 2020). The antimicrobial susceptibility profile of Bcc bacteria identified in Bangkok in recent years is poorly documented. The purpose of this study was to ascertain the pattern of antimicrobial susceptibility of certain Bcc bacteria, specifically *B. cenocepacia*, that were isolated from King Chulalongkorn Memorial Hospital (KCMH), a general and tertiary hospital located in Bangkok, Thailand, between January and April of 2022.

Bacteria in Bcc group carry multiple drug-resistant genes on their chromosomes and plasmids (Mahenthiralingam et al., 2005; Sousa et al., 2011). Thailand has a lower infection rate from Bcc bacteria compared to the US, UK, Australia, and some other Asian countries (Kenna et al., 2017). However, immunocompromised individuals continue to get infected with Bcc bacteria, with *B. cenocepacia* being more frequently found than other species (LiPuma et al., 2002). There are also reports of Bcc transferring genes to other bacteria through horizontal gene transfer (Burtnick et al., 2024; Patil et al., 2017), such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and especially *B. pseudomallei*, a closely related species in the same genus. *B. pseudomallei* causes melioidosis, a common and potentially fatal disease in Thailand. The transfer of antibiotic resistance genes could lead to difficult-to-treat outbreaks, including resistance to common antibiotics like ceftazidime (CAZ), trimethoprim-sulfamethoxazole (SXT), and meropenem (MEM), a carbapenem used to treat melioidosis.

Typically, bacterial species are identified using molecular techniques like Sanger sequencing of the 16s rRNA gene. However, for Bcc, 16s rRNA sequencing cannot clearly distinguish between species and subspecies necessitating the use of alternative species-specific genes. The *recA* or *gyrB* genes are commonly used for Bcc identification (Tabacchioni et al., 2008). This study aims to identify and isolate *B. cenocepacia* using species-specific *gyrB* sequencing and to examine the antibiotic susceptibility patterns. Additionally, we performed whole-genome sequencing to compare the antibiotic resistance genes of *B. cenocepacia* isolates from Thailand with those of reference strain, information from National Center for Biotechnology Information (NCBI) Database.

Research Methodology

Bacterial isolates

Ten Bcc isolates, collected from patients at KCMH between January and April 2022, were used in this study. The strains were isolated by the Microbiology Unit of King Chulalongkorn Memorial Hospital and approved by the Human Ethics Committee (IRB). Isolates were streaked onto on Luria-Bertani (LB) agar (Difco, Lennox) and incubated overnight at 37 °C. Single colonies were then inoculated into LB broth and incubated at 37 °C with shaking at 200 rpm for further growth. The Bcc isolates were preserved in deep freezers at -80 °C in 2 ml cryovial tubes containing 20% glycerol in LB broth until further use.

Species-specific *gyrB* PCR assay

Ten clinical isolates stored at -20°C were revived and their genomic DNA was extracted. A conventional PCR assay targeting a 1,990 bp fragment of *gyrB* gene was performed using a Proflex PCR system (Applied Biosystems). Each 50 µl PCR reaction contained 1x PCR buffer (without $MgCl_2$), 1.5 mM $MgCl_2$, 1U Taq DNA polymerase (Thermo Fisher Scientific), 5% DMSO, 250 µM of each dNTP, 1 µl (20 pmol) of each forward and reverse primer (Table 1), and 25 ng of template DNA.

The thermal cycling conditions were as follows: initial denaturation at 95 °C for 4 minutes, followed by 35 cycles of 95 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 1 minute, with a final extension at 72 °C for 10 minutes. These conditions were adapted from previous study (Mahenthiralingam et al., 2000). PCR products were separated by electrophoresis on a 1.5% agarose gel stained with SYBR® Safe (Invitrogen) and visualized under UV light.

TABLE 1 Primers for PCR amplification of Specific *gyrB* gene

Gene	Primer	Primer sequence (5′ to 3′)	Tm (°C)	Amplicon Size (bp)	Reference
gyrB	gyr1 (forward)	TGGAATTGCCCAATATTATGC	61.8	1,990	(Tabacchioni et al., 2008)
	PC9r (reverse)	TCAGCGCAGCTTGTCGGCCATGC	58.8		

Analysis of *gyrB* sequences

To confirm the nucleotide sequences of the *gyrB* PCR products, Sanger sequencing was performed using a specific primers (Table 2). The resulting sequences were analyzed using BioEdit software and compared to sequences in the NCBI database using the BLASTN function of the BLAST program. Species

assignment was determined based on a sequence similarity threshold of 99% or greater when compared to strains in the NCBI database.

In constructing the phylogenetic tree, a maximum-likelihood phylogenetic tree was generated based on the *gyrB* gene sequences using the General Time Reversible model with G + I rates among sites, and a bootstrap method with 1000 replications in the Molecular Evolutionary Genetics Analysis software, version 7 (MEGA7). The nucleotide sequences were used to build the phylogenetic tree, with *P. aeruginosa* strain PAO1 as the outgroup. The analysis compared to reference Bcc strains including *B. cepacia* strain BC16 (genomovar I), *B. multivorans* strain ATCC17616 (genomovar II), *B. cenocepacia* strain J2315 (genomovar III), and *B. vietnamiensis* strain G4 (genomovar V) (Jin et al., 2020; Mahenthiralingam et al., 2000), and the tree was visualized using iTOL (Jin et al., 2020).

TABLE 2 *gyrB* gene sequencing primers.

Gene	Primer	Primer sequence (5′ to 3′)	Tm (°C)	Position	Reference
gyrB	gyr1 (forward)	F-TGGAATTGCCCAATATTATGC	61.8	340-360	(Tabacchioni et al., 2008)
	PC3r (forward)	F-GAAGTSGCGATGCAGTGGAACGA	64.8	826-848	
	PC6r (reverse)	R-CAGCGGCAGRATWGCCTG	59.4	1410-1393	
	PC9r (reverse)	R-TCAGCGCAGCTTGTCGGCCATGC	58.8	2328-2310	

Antibiotic Susceptibility Testing (AST) of *B. cenocepacia* isolates

Antibiotic Susceptibility Testing (AST) was conducted on five *B. cenocepacia* isolates using three antibiotics: ceftazidime (CAZ), meropenem (MEM), and trimethoprim-sulfamethoxazole (SXT). The Minimum Inhibitory Concentrations (MICs) were determined using the Broth Microdilution Method (BMD), with each antibiotic tested in two technical replicates and three separate biological replicates. The protocol for assessing bacterial resistance to antibiotics followed guidelines set forth by the Clinical and Laboratory Standards Institute (CLSI) for determining antibiotic susceptibility (Clinical and Laboratory Standards Institute [CLSI], 2024.).

The process began with retrieving frozen Bcc isolates, streaking them on LB agar, and allowing overnight incubation at 37°C. A single colony was selected and transferred into 3 ml LB broth and incubated overnight at 37°C with 200 rpm agitation. One percent inoculum from an overnight culture was transferred into 3 ml of Mueller Hinton II Broth Cation-Adjusted (CA-MHB) and incubated with agitation at 37°C until it reached mid-log phase. The mid-log phase culture was diluted in sterile 0.9% sodium chloride solution to an optical density (OD600) of 0.08 and 0.1, corresponding to 0.5 McFarland standard. A 0.1 ml aliquot of this standardized suspension was then inoculated into 4.9 ml of CA-MHB broth for further testing.

Antibiotic susceptibility testing was then performed in 96-well microtiter plates, following CLSI M07 guidelines (CLSI, 2012.). Two-fold serial dilutions of each antibiotics were prepared in sterile CA-MHB broth. Control strains, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, were included for comparison. The plates were incubated at 37°C for 18-20 hours. The MIC breakpoints specific to Bcc bacteria were determined according to CLSI M100 guidelines (CLSI, 2024.). To confirm inhibition of bacterial growth, spectrophotometric measurements at 600 nm were taken. To further assess cell viability and determine the minimum bactericidal concentrations (MBCs), serial dilutions of the cultures were spotted on LB agar. Positive (the standardized suspension without antibiotics) and negative (fresh MHB broth) controls were included to validate the assay.

Whole Genome Sequencing (WGS) and Bioinformatics pipeline

One *B. cenocepacia* isolate was randomly selected for whole genome (WGS). Short-reads sequencing wasperformed using Illumina technology, and long-read sequencing was performed using Oxford Nanopore Technology (ONT).

Oxford Nanopore sequencing

For library preparation, 1 µg of genomic DNA was used with the Rapid Barcoding Kit 96 V14 (SQK-RBK114.96) from Oxford Nanopore Technologies (ONT), Oxford, UK, following the manufacturer's instructions. The sequencing libraries were multiplexed and loaded into the Flow Cell FLO-MIN114 (R10.4.1). DNA samples were sequenced using PromethION (Oxford Nanopore). The raw reads were obtained during a 48-hour run experiment using MinKNOW Version 23.07.12. Raw signals were base called using a super high accuracy model and demultiplexed using Guppy v.5.0.7.

The quality of the raw long reads was assessed using Nanoplot (De Coster et al., 2018) (Version 1.41.0) and processed with Porechop (Bonenfant et al., 2023) (Version 0.2.4), an adapter trimmer for Oxford Nanopore reads, to eliminate adapter and barcode sequences. Long reads were filtered for

quality using Filtlong (Version 0.2.1), applying a minimum length threshold and a minimum quality score of greater than 1000 bp and Q score > 8, respectively (https://github.com/rrwick/Filtlong).

Illumina sequencing

Paired-end sequencing of genomic DNA was performed on a MiSeq platform (Illumina, San Diego, CA, USA) using V2 chemistry with 250x2 Paried-end reads (Behzadi et al., 2021; Nokchan et al., 2021). Trimmomatic (Version 0.38.1) was used to process the short reads in order to remove adapter and barcode sequences, correct mismatches in overlaps, and filter low-quality reads (Quality Score (Q) > 30) (Bolger et al.). FastQC (Version 0.74; https://github.com/s-andrews/FastQC) was used to evaluate the quality of the first brief reads.

***B. cenocepacia* complete genome**

Unicycler (Version 0.5.0) was used for de novo hybrid assembly, integrating long and short readings with default parameters (Wick et al.). The quality of the genome was determined using QUAST (Version 5.0.2)(Gurevich et al., 2013). The assembled genome sequence was annotated by Prokka (Seemann). Using the Proksee, the assembled contigs from the combined short read and long read sequencing were circularized before being compared to the reference genome (*B. cenocepacia* J2315) (Wallner et al., 2014). To find antibacterial gene, contigs were screened using ABRicate (Version 1.0.1) (https://github.com/tseemann/abricate) using ABRicate with the CARD RGI and NCBI databases in Galaxy for identifying antimicrobial resistance genes (Galaxy, 2024).

Results and Discussion

Species-specific *gyrB* sequence analysis

Using species-specific *gyrB* gene sequencing, ten Bcc isolates collected from patients at King Chulalongkorn Memorial Hospital, Thailand, were identified. The resulting nucleotide sequences were used to construct a phylogenetic tree. The phylogenetic tree in Figure 1 revealed that the clinical isolates clustered into three main clades alongside reference strains of Bcc. The first clade included *B. cenocepacia* strain J2315 (genomovar III) and encompassed SCBC65, SCBC75, SCBC77, SCBC86, and SCBC87.The second clade consisted of *B. cepacia* strain BC16 (genomovar I), along with isolates SCBC76, SCBC91, and SCBC95. The third clade contained B. vietnamiensis strain G4 (genomovar V), with isolate SCBC96 positioned within this clade. There are no strains in the Bcc sample group within the clade of genomovar II. *P. aeruginosa* strain PAO1 was used as an outgroup to root the tree and was clearly distinct from the Bcc strains.

Figure 1 Phylogenetic tree of the *gyrB* gene, illustrating relationships among 10 Bcc isolates obtained from patients at King Chulalongkorn Memorial Hospital, Thailand, compared to reference Bcc strains including *B. cepacia* BC16 (genomovar I), *B. multivorans* ATCC17616 (genomovar II), *B. cenocepacia* J2315 (genomovar III), and *B. vietnamiensis* G4 (genomovar V). The tree was constructed using MEGA7 and further customized for presentation with iTOL, with *P. aeruginosa* strain PAO1 serving as the outgroup.

Assessment of Antibiotic Sensitivity

Broth Microdilution testing demonstrated that ceftazidime (CAZ), meropenem (MEM), and trimethoprim-sulfamethoxazole (SXT) effectively inhibited the growth of all tested *B. cenocepacia* isolates. Table 3 displays the minimum inhibitory concentration (MICs) of each antibiotic for each strain, as well as the with the corresponding CLSI breakpoints (Table 3) for *B. cepacia* complex.

Table 3 Minimum Inhibitory Concentration (MIC) breakpoints

	MIC breakpoints (µg/mL)		
	S	I	R
Ceftazidime	≤ 8	16	≥ 32
Meropenem	≤ 4	8	≥ 16
Trimethoprim/Sulfamethoxazole	≤ 2/38	-	≥ 4/76

Source: CLSI M100 (*CLSI. Performance Standards for Antimicrobial Susceptibility Testing, 34th ed. CLSI guideline M100. Clinical and Laboratory Standards Institute; 2024.*)

Table 4 Minimum Inhibitory Concentration (MIC) of antibiotics against *B. cenocepacia* isolates (n=5).

	MIC range (µg/mL)		
	Ceftazidime	Meropenem	Trimethoprim/Sulfamethoxazole
SCBC65	4-8	8	2/38
SCBC75	4	4	1/19
SCBC77	4	4	1/19
SCBC86	4-8	1-2	2/38
SCBC87	2-4	4	1/19

All five *B. cenocepacia* isolates tested were susceptible to the antibiotics commonly used for treating Bcc bacterial infection at KCMH. As shown in Table 4, the MICs varied among strains, demonstrating that the tested antibiotics remain effective against *B. cenocepacia* strains. This information is crucial for guiding therapeutic decisions in clinical settings.

Whole Genome Sequencing and Antibiotic Resistance Gene Analysis of *B. cenocepacia* Strain SCBC75

Hybrid whole genome sequencing was used to assemble the complete genome of *B. cenocepacia* SCBC75, revealing its chromosomal structures and plasmids (Figure 2). The genome of *B. cenocepacia* SCBC75 was compared to the reference strain J2315 (represented in yellow in Figure 2) to identify genomic differences. The comparison revealed substantial genomic differences between the two strains, particularly in the plasmid sequences. These differences likely reflect the higher rate of gene exchange in plasmids

compared to chromosomes, as well as the distinct geographical and environmental origins of the two strains. We then identified antibiotic resistance genes (shown in red in Figure 2) on all three chromosomes and plasmids of *B. cenocepacia* SCBC7 5. Using ABRicate with the CARD RGI and NCBI databases, we identified resistance genes, as detailed in Table 5. On chromosome 1, we detected genes with 81.29% and 88.24% identity to *amrA* and *amrB*, respectively. These genes encode component of the AmrAB-OprM multidrug efflux complex, which confers resistance to aminoglycosides such as amikacin and gentamicin. The *amrA* gene also corresponds to a locus in *P. aeruginosa* PAO1 and LESB58, suggesting a genomic relationship between *B. cenocepacia* and *P. aeruginosa* (Jassem et al., 2014; Westbrock-Wadman et al., 1999).

On chromosome 2, we identified the resistance gene *omp38* with 80% identity. Heterologous expression of *B. pseudomallei* Omp38 (BpsOmp38) in Omp-deficient *E. coli* host cells reduces permeability, lowering susceptibility to penicillin G, cefoxitin, ceftazidime, and imipenem. Mutation in this gene could decrease the uptake of carbapenems, cephalosporins, cephamycins, and monobactams in *Burkholderia* (Aunkham et al., 2014; Siritapetawee et al., 2004).

Table 5 Antibiotic Resistance Genes Identified in *B. cenocepacia* SCBS75.

Located	Gene	%Coverage	%Identity	Product	RESISTANCE
Chromosome1	*amrA*	93.67	81.29	Efflux pump subunit of the AmrAB-OprM multidrug efflux complex	aminoglycoside
	amrB	99.23	88.24	The membrane fusion protein of the AmrAB-OprM multidrug efflux complex	aminoglycoside
Chromosome2	*omp38*	95.63	80.44	Permeability channel for the antibiotics penicillin G, cefoxitin, ceftazidime, and imipenem	carbapenem, cephalosporin, cephamycin, monobactam
	opcM	99.93	96.36	Outer membrane factor protein found in *B.cepacia*. It is part of the CeoAB-OpcM complex	aminoglycoside, fluoroquinolone

	ceoB	100	98.12	Cytoplasmic membrane component of the CeoAB-OpcM efflux pump	aminoglycoside, fluoroquinolone
	ceoA	99.92	96.97	Periplasmic linker subunit protein of the CeoAB-OpcM efflux pump	aminoglycoside, fluoroquinolone
	penA	99.89	89.79	PenA family class A beta-lactamase	BETA-LACTAM

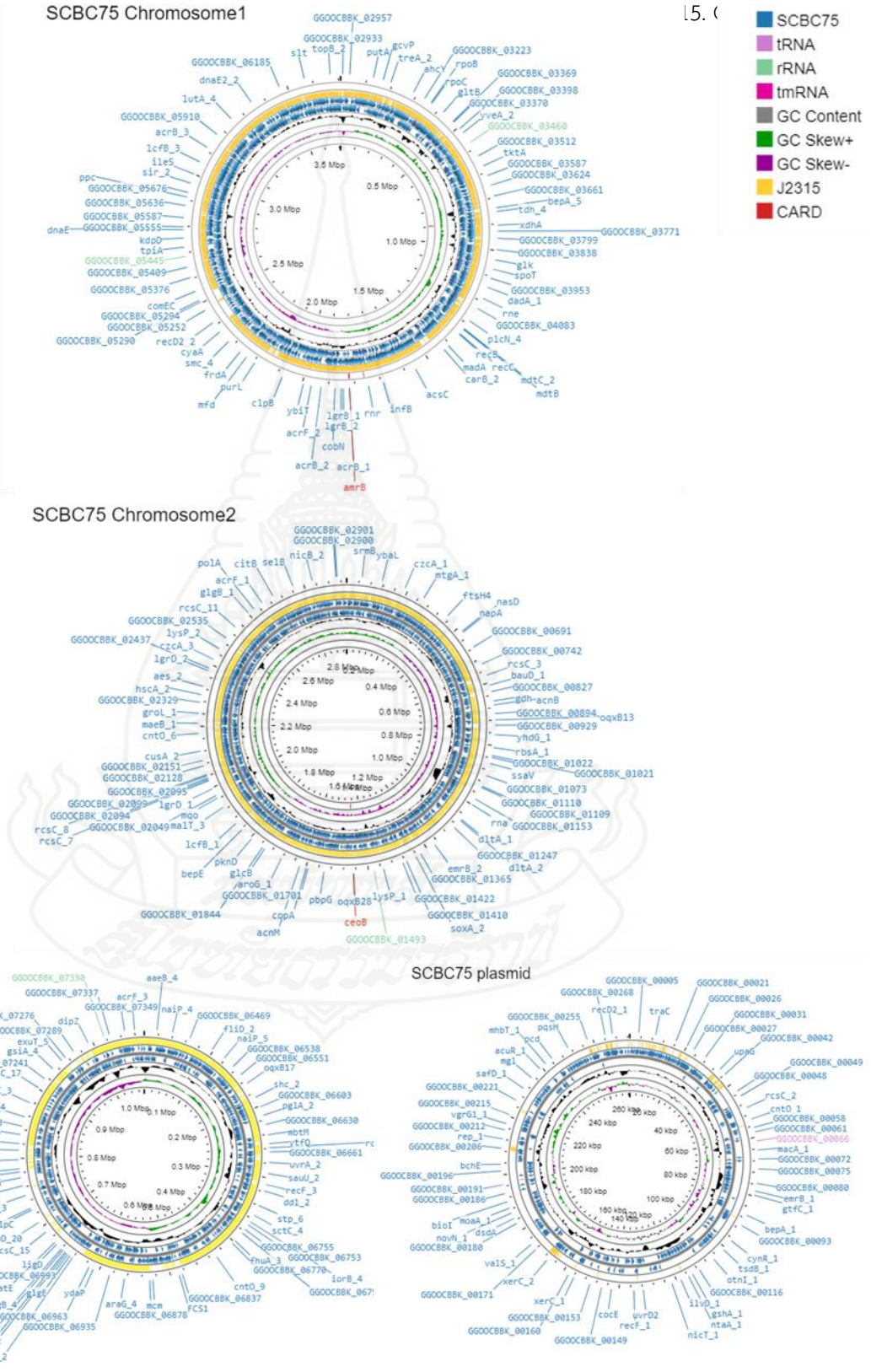
Using ABRicate with the CARD RGI and NCBI databases in Galaxy platform (Galaxy, 2024)

Additionally, we found the genes *opcM*, *ceoA*, and *ceoB*, with 96.36%, 96.96%, and 98.12% identity, respectively. These genes are involved in synthesizing protein factors and subunits ofthe CeoAB-OpcM complex, another multidrug efflux pump in the *B. cepacia* complex thatexpels aminoglycosides and fluoroquinolones, contributing to resistance against these drug classes (Hussin et al., 2024).

Comparison with the NCBI database revealed a sequence with 89.79% identity to the *penA* of *B. pseudomallei*. This gene is known to confer resistance to beta-lactam antibiotics through the production of a class A beta-lactamase, an enzyme that hydrolyzes the amide bond in beta-lactam antibiotics such as ceftazidime. This enzyme utilizes an active site serine residue its catalytic activity (Devanga Ragupathi & Veeraraghavan, 2019; Rholl et al., 2011).

This whole-genome sequencing (WGS) study focused on identifying potential antibiotic resistance genes in a single *B. cenocepacia* isolate from Thailand, rather than investigating the specific mechanisms by which each gene contributes to resistance. Our goal is to establish a foundational knowledge base for future research on this pathogen in our region, particularly regarding the expression and regulation of antibiotic resistance genes.

Figure 2 Comparati 5. (



representation of the complete genomes of *B. cenocepacia* SCBC75 (inner ring) and reference strain J2315 (outer ring).

- [Blue]: Genomic regions of SCBC75, shown as two regions with arrows indicating the direction of gene functions.
- [Yellow]: Genomic regions of J2315.
- [Red]: Antibiotic resistance genes identified in SCBC75 (CARD database).

Additionally, other details within the genomes of *B. cenocepacia* SCBC75 are shown, such as:

- [Gray]: GC content.
- [Dark green]: GC skew+
- [Purple]: GC skew-
- [Lilac]: tRNA regions.
- [Light Green]: rRNA regions.
- [Magenta]: tmRNA
- [Others]: Various genomic featres as applicable.

Conclusion

This study focuses on *B. cenocepacia*, which is the most commonly found species among all species in the complex (LiPuma et al., 2002) and it is also the species that significantly increases the risk of mortility in those infected compared to other strains in *B. cepacia* complex (Syed & Wooten, 2021). By employing the established *gyrB* gene sequencing method, we identified five *B. cenocepacia* strains among ten Bcc isolates from King Chulalongkorn Memorial Hospital, Thailand. These strains were susceptible to commonly used antibiotics, suggesting that current treatment regimens remain effective. The predominance of *B. cenocepacia* underscores its importance as a cause of Bcc infections at KCMH. However, the presence of other species, such as *B. cepacia* and *B. vietnamiensis*, highlights the need for accurate species identification to guide appropriate treatment decisions.

The antibiotic resistance profiles of the five *B. cenocepacia* strains isolated and tested against the three antibiotics currently used for treating bacterial infections at King Chulalongkorn Memorial Hospital revealed that all strains remained susceptible to these drugs.

Whole-genome sequencing (WGS) of randomly selected strain, *B. cenocepacia* SCBC75, a clinical isolate from Thailand, revealed significant genomic diversity compared to the reference strain J2315. Notably, we identified several genes associated with antibiotic resistance mechanisms, including *penA* (beta-lactam resistance) and two families of efflux pump genes (*amr* and *ceo*) conferring resistance to aminoglycosides and fluoroquinolones, respectively. The presence of these genes in a clinical isolate

underscore the potential for the emergence of multidrug-resistant *B. cenocepacia* strains, posing a challenge for treatment and infection control.

This study represents one of the few investigations employing molecular techniques to identify and differentiate strains of the *B. cepacia* complex, specifically focusing on *B. cenocepacia*, which was found to be more prevalent than other species. The genomic analysis of *B. cenocepacia* SCBC75 aims to enhance the understanding of genes associated with antibiotic resistance mechanisms in this strain, potentially paving the way for future research into its resistance profiles.

Suggestion

Research on *B. cenocepacia* in Thailand is still limited, highlighting the need for continued investigation into its prevalence, virulence, and antibiotic resistance mechanisms. While this study provides valuable insights into the genomic features and antibiotic resistance genes of a clinical isolate, it was constrained by limited time, sample size, and budget.

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