Evaluation of the Synergistic Antibacterial Effects of Rifampicin-based Combination Therapies for the Management of Infections due to Carbapenem-Resistant *Acinetobacter baumannii*

Lois Chinwe Nwabor

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Master of Science in Biomedical Sciences

Prince of Songkla University
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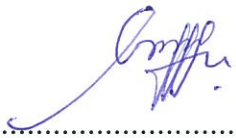

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

.....Signature

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Candidate

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Abstract

The increasing spread of carbapenem-resistant *Acinetobacter baumanii* (CRAB) is critical to public health due to the lack of treatment options and increased mortality rate. Herein, the synergistic and bactericidal effects of rifampicin in combination with conventional antibiotics were evaluated, and the molecular pathways of antibiotic resistance were predicted using bioinformatics and whole-genome sequencing. Phenotypic analysis including efflux pump detection and antibacterial activity of combination were evaluated against established biofilm cells using carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and MTT assay, respectively.

From the evaluations, about 89% of the 218 CRAB clinical isolates tested in the study showed resistance to rifampicin at zones of inhibition ≤16 mm, 9% were intermediate (17–19 mm), and 1% were susceptible (≥20 mm). The antibiotic-resistant profiles of the isolates were investigated in 31 representative clinical isolates. A total of (22/31) 71% and 94% (29/31) isolates demonstrated susceptibility to tigecycline and minocycline, respectively. The isolates showed multidrug resistance and exhibited a 100% resistance to gentamycin or tobramycin at Minimum inhibitory

concentration (MIC) ≥ 1024 µg/mL, and chloramphenicol at ≥ 16µg/mL. Five isolates out of 20 were resistant to colistin at MIC = 4 µg/mL whereas 15 were intermediate at MIC=2 µg/mL. Combination therapy of rifampicin slightly improved antibiotic potency with synergism in 10/31, 7/31, 2/31, 4/31, 5/31, and 8/9 when combined with imipenem, meropenem doripenem, tigecycline, minocycline, and colistin respectively.

In addition, time-kill kinetic revealed a bactericidal effect at higher concentrations and a bacteriostatic at lower concentrations. The combination of rifampicin plus imipenem and doripenem was bactericidal against TR123 at 1/4 MIC of rifampicin and 1/4MIC of doripenem and imipenem. Rifampicin combined with tigecycline or minocycline was bactericidal at 1/4MIC rifampicin plus 1/4 MIC of the antibiotics against TR131 out of three representative isolates. Rifampicin combination with tigecycline disclosed a 2-2.5 log reduction in CFU at all the combined concentrations including at 1/4 MIC rifampicin and 1/4 MIC of tigecycline. Rifampicin plus ciprofloxacin resulted in a 99% killing against TR023 out of three isolates indicating a bactericidal activity at MIC rifampicin plus 1/2 MIC ciprofloxacin, 1/2 MIC rifampicin plus 1/2 MIC ciprofloxacin and 1/2 MIC rifampicin plus 1/4 MIC ciprofloxacin. Rifampicin plus chloramphenicol or trimethoprim-sulfamethoxazole combinations were not effective at MIC and sub- Inhibitory concentrations among all the isolates used. The combination therapy of rifampicin and fosfomycin disclosed a bacteriostatic effect against two representative isolates. Notably, rifampicin with colistin exhibited bactericidal activity in three out of four representative isolates (PT046, TR069, and TR082) at 1/4 MIC rifampicin plus 1/4 colistin.

Antibacterial resistant mechanism assessment indicated a 4-fold reduction in the MIC of rifampicin in the presence of the efflux pump inhibitor CCCP

in isolates SK056 and SK067 out of the 15 tested isolates. Biofilm viability test by MTT assay revealed a dose-dependent decrease of cell viability of established bacterial biofilm at 4 MIC rifampicin + 2 MIC carbapenems with a percentage reduction of 44–75%, compared with monotherapies at 16 MIC. The pan-genomic study of the isolates demonstrated a progressive evolution with 58% of accessory genes in the matrix. Seven of the ten sequenced isolates were of sequence type 2 (ST2), while one isolates each belongs to ST164, ST16, and ST25. Furthermore, 11 plasmids, 34 AMR genes, and 65 virulent genes were predicted to confer MDR. The *bla*$_{\text{OXA-23}}$ *bla*$_{\text{ADC-25}}$, *bla*$_{\text{OXA-66}}$, *bla*$_{\text{PER-7}}$, *aph(6)-Id*, *armA*, and *arr-3* were prevalent among the isolates. Sequence alignment of the bacteria genome to a reference strain revealed a deleterious mutation in the *rpoB* gene in 4 out of 29 isolates. Colistin-resistance-associated mutation on the PmrB and PmrC (two-component system), LPS biosynthetic protein *lpxD*, *emrA*, and *emrB* genes were detected among the five isolates that demonstrated resistance to colistin.

This research emphasizes the specificity of isolates to antibiotics and suggests that the rifampicin combination with colistin, tigecycline, minocycline, imipenem, meropenem, and doripenem may be a potential treatment option for the management of CRAB isolates with low rifampicin minimum inhibitory concentration. It also demonstrates that the genotypic and phenotypic characterization of antimicrobial resistance (AMR) in CRAB clinical isolates may lessen the burden of AMR surveillance.

Keywords: *Rifampicin-resistant and carbapenem-resistant Acinetobacter baumannii;combination therapy; antibiofilm; antibacterial, whole genome sequencing, antimicrobial resistance, efflux pump, rifampicin.*

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Lois Chinwe Nwabor

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LIST OF ABBREVIATIONS

ABC = ATP Binding Cassette

AAC = Aminoglycosides Acyltransferases

ACICU_0071 =ATPase

ACICU_0072 =Protein Tyrosine Phosphate

ACICU_0073 = Periplasmic Protein

ACICU_0074 = UDP-N-acetyl-D-Mannosaminuronate Dehydrogenase

ACICU_0075 = Nucleoside-Diphosphate Sugar Epimerase

ACICU_0076 = Pyridoxal Phosphate-Dependent Enzyme

ACICU_0077 = CMP-N-Acetylneuraminic Acid Synthetase

ACICU_0078 = Spore Coat Polysaccharide Biosynthesis Protein (glycosyltransferase)

ACICU_0079 = Acetyltransferase

ACICU_0080 = Sialic Acid Synthase

ACICU_0086 = Glycosyltransferase

ACICU_0087 = Sugar Transferase

ACICU_0088 = UDP-Glucose Pyrophosphorylase

ACICU_0089 = UDP-Glucose-6-Dehydrogenase

ACICU_0091 = UDP-Glucose-4-Epimerase Pyrophosphorylase

ACICU_0092 = Phosphomannomutase

ACBC = Acinetobacter Calcoaceticus Baumannii Complex

AHL = Acyl Homoserine Lactone

AMR = Antimicrobial Resistance/ Resistant

ANT = Aminoglycoside Adenyltransferases

APH = Aminoglycoside Phosphotransferases

ARR = ADP-Ribosyl-Transferase

BAP = Biofilm Associated Protein

Bfm = Biofilm Formatiom

Bfms = Biofilm Formation and Cellular Morphology

BGI = Beijing Genomic Institute

BSI = Blood Stream Infection

CAP = Community-Acquired Pneumonia

CCCP = Carbonyl Cyanide Mchlorophenylhydrazone

CDC = Center for Disease Control and Prevention

CGE = Center For Genomic Epidemiology

CSU = Chaperone-usher

CIP = Ciprofloxacin

CFU = Colony Forming Unit

CPH = Chloramphenicol

CKD = Chronic Kidney Disease

CRAB = Carbapenem-Resistant *Acinetobacter baumannii*

COL = Colistin

CTX-M = Cefotaxime β-Lactamase

DOR = Doripenem

DNA = Deoxyribonucleic Acid

ESKAPE = *Enterococcus faecium, Staphylococcus aereus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter species.*

ESBL = Extended Spectrum β-lactamases

FICI = Fractional Inhibitory Concentration Index

FOS = Fosfomyxin

GEN = Gentamicin

GNB = Gram Negative Bacteria

HemO = Hemophore

ICU = Intensive Care Uniit

IMP = Imipenem or Imipenemase

IS = Insertion Sequence

LEV = Levofloxacin

LPS = Lipopolysaccharides

MALDI-TOF MS = Matrix-Assisted Laser Desorption/Ionisation-time of Flight Mass spectrometry

MATE = Multi-Drug and Toxic Compound Extrusion

MDR = Multi-drug resistant

MEM = Meropenem

MFS = Major Facilitator Superfamily

MGE = Mobile Genetic elements

MHB = Mueller Hinton Broth.

MHA = Mueller Hinton Agar.

MIC = Minimal Inhibitory Concentration

MIN = Minocycline

MLST = Multilocus Sequence Typing

NARST = National Antimicrobial Resistance Surveillance Centre, Thailand

NCBI = National Centre for Biotechnology Information

ND = Not Determined

NDM = New Delhi Metallo-β- Lactamase

OmpA = Outer Membrane Protein A

OMV = Outer Membrane Vesicles

OXA = Oxacillinase

PA = Pattani

PACE = Proteobacterial Antimicrobial Compound Family

PDR = Pan-drug Resistance

PBPG = Penicillin-Binding Protein

Plc = Phospholipase C

plcD = Phospholipase D

POL = Polymycin

PNAG = β-(1-6)- Poly-N-acetyl -D-glucosamine

PT = Phatthaling

RNA = Ribonucleic Acid

RND = Resistance Nodulation Division

RPOB = RNA Polymerase β-subunit

RTI = Respiatory Tract Infection

SEM = Scanning Electron Microscopy

SHV = Sulfhydryl Variable

SMR = Small Multi-Drug Resistant Family

SNP = Single Nucleotide Polynorphisim

ST- Sequence Type or Satun

TEM = Temoniera

TIG = Tigecycline

TOB = Tobramycin

TMP/SMZ = Trimethoprim- Sulphamethaxazole

TR = Trang

TSA = Tryptic Soya Agar

TSB = Tryptic Soya broth

UTI = Urinary Tract Infection

VAP = Ventilator-Associated-Pneumonia

VIM = Verona Integron-Encoded Metallo-B-Lactamases.

VFDB = Virulence Factor Database

WGS = Whole Genome sequencing

XDR = Extensive Drug Resistance

CHAPTER 1

INTRODUCTION

Background of the study and rationale

Antibiotic resistance among clinically relevant Gram-negative bacteria (GNB) has become a global problem and a major challenge to public health. GNB naturally possess a structural barrier that limits the influx of antibiotics and can adapt to different microenvironments with the acquisition of virulent factors. In the past, carbapenem-resistant *Acinetobacter baumannii* (CRAB) was linked with increased morbidity and mortality rate of about 26-60% (Xiao et al., 2017) , according to CDC an estimated $ 281 million in healthcare spending, 8500 hospitalized patients, and 700 deaths (Colquhoun & Rather, 2020) annually in the USA . More recently, *A. baumannii* accounts for about 54% of mortality in the intensive care unit (ICU) among other pathogens (Mirzaei et al., 2020) and has been shown to infect approximately 1,000, 000 persons per annum (Vrancianu, Gheorghe, Czobor, & Chifiriuc, 2020). The CDC 2020 report listed carbapenem resistance *Acinetobacter* species as second among the 18 most alarming threats of antimicrobial resistance, amounting to a health burden of about 4.6 billion per annum in the USA. In addition, since the COVID-19 pandemic *A. baumannii* coinfection has resulted in a 100% mortality rate (Lima, Brito, & da Cruz Nizer, 2020). Data collated from the National Antimicrobial Resistance Surveillance center Thailand (NARST) in 2020 reported 70.1% and 69% resistance to imipenem and meropenem, respectively (Kiddee et al., 2019).

As the burden of antimicrobial resistance progresses, most formerly reliable antibiotics have become ineffective against GNB, resulting in multidrug resistance (MDR). Carbapenems are preferred for the management of infections caused by GNB because they are less toxic, potent, and with a broad spectrum of activity. However, the indiscriminate usage in humans and agricultural practices resulted in the development and rapid spread of resistance among both environmental and clinical isolates (Aminov, 2010; Meletis). *A. baumannii* is associated with infections such as bloodstream infection (BSI), respiratory tract infection (RTI), and urinary tract infection (UTI) (Cai et al., 2017a; Tilahun, Gedefie, Bisetegn, & Debash, 2022). Studies suggested that *A. baumannii* infections are common among patients on a mechanical ventilator, and other forms of medical intubations (Duman, Kuzucu, Ersoy, & Otlu, 2020).

Given that most coronavirus disease 2019 (COVID-19) patients are often placed on medical devices, *A. baumannii* biofilm has been reported to play a vital role in the increased severity of infections among patients in the ICU (Zhang, Li, Yu, & Wang, 2020). With the closeness of cells in the biofilm environment, research has shown that genetic materials are transferred among cells leading to mutation and integration of materials carried by mobile genetic element (MGE) (Soucy, Huang, & Gogarten, 2015). The activity of the *emrA/emrB* efflux pump genes has been associated with increased biofilm formation and colistin resistance in *A. baumannii* (Lin, Lin, & Lan, 2020). Recently, studies have highlighted different mechanisms of resistance of *A. baumannii* to antibiotics, including carbapenems, fluoroquinolone, aminoglycosides, and cephalosporin (Kadri et al., 2018; Logan, Gandra, Trett, Weinstein, & Laxminarayan,

2019). Whole-genome sequencing (WGS) of CRAB identified the *arr-3* gene which might confer resistance to rifampicin (Chukamnerd et al., 2022a). Furthermore, the synthesis of hydrolytic enzymes (carbapenemases) (Peleg & Hooper, 2010), antibiotic exclusion through efflux pumps or upregulation of efflux pump regulatory gene, the mutation of outer membrane protein (Livermore, 2012), the presence of plasmid genes, and the ability to form biofilms on hospital wares and devices (Ebrahimi, Sisakhtpour, Mirzaei, Karbasizadeh, & Moghim, 2021), are common antimicrobial-resistant mechanisms. The presence of OXA-type carbapenemases genes including the $bla_{\text{OXA-23}}$, and over-expression of $bla_{\text{OXA-51}}$ mediates resistance to carbapenems (Ibrahim, Ibrahim, Ibrahim, Hamid, & Alaziz, 2022).

The resistance of *A. baumannii* to carbapenems has led to the use of alternative treatment regimens including colistin, tigecycline, and minocycline, which are cytotoxic (Bartal, Rolston, & Nesher, 2022). As well as the repurposing of antibacterial agents that are not indicated for the treatment of GNB, such as vancomycin and fosfomycin. Combination therapy is one of the numerous strategies currently employed for the treatment of CRAB (Ni et al., 2016). Recently, some synergistic mechanisms were proposed to effectively manage antimicrobial resistance pathogens which include sequential blockade, enhanced bioavailability, inhibitor suppression, pathway inhibition, and mutual stabilization which employ a combination of regimens (Sullivan, Delgado, Maharjan, & Cain, 2020).

In previous studies, rifampicin combination therapy with colistin enhanced the antibacterial activity of the cationic peptide against *A. baumannii* (Leite et

al., 2016). The combination of rifampicin with Polymyxin B exhibited bactericidal killing of extensive drug resistance (XDR) *A. baumannii* isolates (Teo et al., 2015). Adjunctive rifampicin therapy with colistin and tigecycline also demonstrated synergism against *A. baumannii* isolates (Bai et al., 2015). Although various antibiotic combinations have demonstrated synergistic activities, the effects of rifampicin-based combinations have only been investigated with colistin and a few other antibiotics. Hence there is insufficient data on the activities of rifampicin with antibiotics classes such as aminoglycosides, carbapenems, glycylcycline, fluoroquinolones trimethoprim-sulphamethoxazole, and fosfomycin against *A. baumannii* isolates**.**

1.2 Research problems

- The alarming increase in resistance of *A. baumannii* to antibiotics and its rising mortality in the health care sector have become a source of concern and a threat to public health.
- The failure of the mono-therapeutic administration of antibiotics to effectively eradicate resistant strains and the general shortage of treatment options is worrisome.
- The increase in the concentration of antibiotics in the environment may lead to the emergence of more virulent mutants of *A. baumannii.*
- Increased concentrations of antibiotics may overwhelm cellular homeostasis and impose cytotoxic effects.

1.3 Research gap

Since the identification of CRAB, there have been no new antibiotics with the potency to treat CRAB except for tigecycline. The synergistic activity of rifampicin and colistin with few antibiotics have been studied on MDR Gram-negative organisms and have been reported to exert therapeutic relevance. However, insufficient data on the activities of rifampicin with antibiotics classes such as aminoglycosides (amikacin, gentamycin), carbapenems (imipenem, meropenem, doripenem), tigecycline, ciprofloxacin, levofloxacin, trimethoprim-sulfamethoxazole, fosfomycin and vancomycin against *A. baumannii* clinical isolates is lacking for Southern Thailand.

1.4 General objective

- This work aims to investigate the *in-vitro* outcome of combination therapies of rifampicin with conventional antibiotics for the management and treatment of carbapenem-resistant *A. baumannii.*

1.5 Specific objectives

- To investigate the incidence of *A. baumannii* resistance to carbapenems
- To conduct an antibiogram profiling of CRAB.
- To evaluate the *in vitro* antimicrobial efficacy of combinations of rifampicin with other inactive antibiotics against the isolates.
- To study the mechanisms of resistance through efflux pump detection, biofilm study WGS investigation for the presence of resistant mediating genes.

1.6 Significance/benefit of the study

- The study may provide a reliable treatment option for the management of CRAB in Southern Thailand and may reduce the global threat posed by carbapenem resistance in *A. baumannii*.
- It explored the different mechanisms of resistance and detected resistance genes for better decision-making during treatment administration.
- It also served as an addition to medical research literature.

CHAPTER 2

LITERATURE REVIEW

2. 1 Emergence of resistance in Gram-negative bacteria (GNB)

Most Gram-negative bacteria (GNB) have developed resistance to various classes of antibiotics previously effective as a prophylactic or therapeutic regimen. Antimicrobial resistance in these organisms has been classified into multidrug resistance (MDR), extensive drug resistance (XDR), and Pan-drug resistance (PDR) based on the extent and antimicrobial resistance index. MDR implies resistance to at least one antibacterial agent in three or more classes of antibiotics whereas XDR describes resistance to at least one antimicrobial agent in all and susceptibility in two or fewer antimicrobial classes, and PDR is defined as resistance to all classes of antimicrobial agents (Kheshti, Pourabbas, Mosayebi, & Vazin, 2019). The World Health Organization (WHO) 2017 priority list of antibiotic-resistant pathogens defined three groups (critical priority, high priority, and medium priority), based on severity and urgency. Antimicrobial-resistant GNB are listed in the top tier of critical and high priority including *A. baumannii, Pseudomonas aeruginosa, Klebsiella pneumonia* (Magiorakos et al., 2012). The ability of GNB to resist diverse classes of antibiotics has been linked to alterations in the predominant structural features such as lipopolysaccharide (LPS), porin proteins, and capsules in the outer membrane. The LPS limits the passage of hydrophobic molecules such as chloramphenicol and aminoglycosides, while the outer membrane protein (porin) permits selective permeation of only small hydrophilic molecules such as β-lactams, and the capsule acts as a shade against lysing chemicals.

Increase in porin expression, upregulation of the efflux pump, formation of biofilm, and secretion of a hydrolyzing enzyme, have been identified as a mechanism of acquired resistance in GNB (Magiorakos et al., 2012; Subhadra et al., 2018; Tantisuwanno et al., 2021; Weiner et al., 2016). Antimicrobial resistance in GNB can be classified into intrinsic, adaptive, and acquired resistance (Iskandar et al., 2022; Reygaert, 2018).

Some inherent features in GNB cells act as a shield from toxic substances including antimicrobial agents. The outer membrane components such as LPS and porins confer intrinsic resistance to antibiotics through selective permeation limiting the entrance of molecules (Arzanlou, Chai, & Venter, 2017). Exposure to sublethal concentrations of antibiotics has been indicated has one of the factors causing the development of resistance in pathogenic organisms (Arzanlou et al., 2017). Pathogenic organisms develop resistance through the acquisition of adaptive abilities to survive unfavorable conditions in the microenvironment (He et al., 2015). Iron acquisition is an adaptive resistance mechanism revealed in GNBs such as *P. aeruginosa* to help promote cellular viability (Bonneau, Roche, & Schalk, 2020). The ability to acquire new functions via horizontal gene transfer (HGT) has been identified to confer resistance to treatment regimens in bacteria isolates. Plasmid-mediated resistant gene transfer has been demonstrated in GNB (Jia, Chen, Wang, & Ruan, 2019). Gene mutation is identified as a form of acquired resistance and may result in the growth of an organism in the presence of antibiotics of which susceptibility was formerly reported (Iskandar et al., 2022; Partridge, 2015). More studies have revealed the wide spread of resistant genes in clinically relevant pathogens reported to originate from the mobilized colistin-resistant

gene (*mcr*) (Liu et al., 2016) in GNB obtained from many countries (Shen et al., 2020). Similarly, the wide spread of the carbapenem-resistant gene $bla_{\text{NDM-1}}$ gene has been detected in several GNB (Hasan, Perveen, Olsen, & Zahra, 2014).

2.2 Acinetobacter calcoaceticus baumannii complex (Acbc)

Acinetobacter calcoaceticus baumannii complex is made of species of *Acinetobacter* that possess closely related phenotypes which make it difficult to differentiate among members of the complex. Some identified clinically relevant species belonging to the complex include *A. baumanni, A. calcoaceticus, A. noscomialis,* and *A. pitti* (Marí-Almirall et al., 2019).

2.2.1 *Acinetobacter baumannii*

A. baumannii is a Gram-negative aerobe, coccobacillus, non-fermentative, oxidase negative, nitrate negative, catalase positive, belonging to the ESKAPE pathogen (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A. baumannii, Pseudomonas aeruginosa,* and *Enterobacter species*.) *A. baumannii* has emerged as a biological superbug responsible for most hospital-acquired infections and recently has been found in community-acquired infections (Jia et al., 2019). Antibiotic resistance in this organism is associated with a high level of acquisition of resistant determinants via gene transfer, mutation, overexpression of efflux pumps, synthesis of antibiotics modifying enzymes, and membrane alterations. *A. baumanniii* promotes the colonization of opportunistic pathogenic organisms and mediates a breakdown of the body system in most immuno-compromised people, especially ICU patients. Information on respiratory

tract infection revealed 80% CRAB and 90% MDR in 97 respiratory tract samples collected from Southern Vietnam (Hoang Quoc et al., 2019). As investigations remain ongoing, records of patients recovering from ventilator-associated pneumonia (VAP) show that the rate of MDR, XDR, and PDR *A baumannii* was 13.3%, 68.3%, and 18.3 % respectively (Jia et al., 2019). A recent meta-analysis reported that the resistance phenotype of this pathogen has spread across 29 countries with cases of VAP and hospital-acquired infection (Lim, Abidin, Liew, Roberts, & Sime, 2019). Patients affected by disease conditions like hypertension, chronic obstructive pulmonary disease, chronic renal failure, long periods of ICU stay, history of organ failure, and low blood oxygenation level are the most at risk of *A. baumannii* infection (Čiginskienė, Dambrauskienė, Rello, & Adukauskienė, 2019). Most antibiotics are now ineffective against *A. baumannii* as indicated in the treatment records of patients with failed therapies such as intracranial *A. baumannii* infection (Čiginskienė et al., 2019).

Recently, the emergence of a sporadic carbapenem resistance clone of sequence type 880 bearing a plasmid-encoded gene $bla_{\text{oxa-72}}$ gene was captured to be responsible for *A baumannii* community-acquired pneumonia (CAP) in China (Jia et al., 2019). Other studies have shown the prevalence of CAP initiated by *A. baumannii* in other countries and continents including Australia, Asia, China, Oceanic, Taiwan, and Thailand (Wong et al., 2017).

2.3 Mechanisms of antibiotic resistance in *A. baumannii*

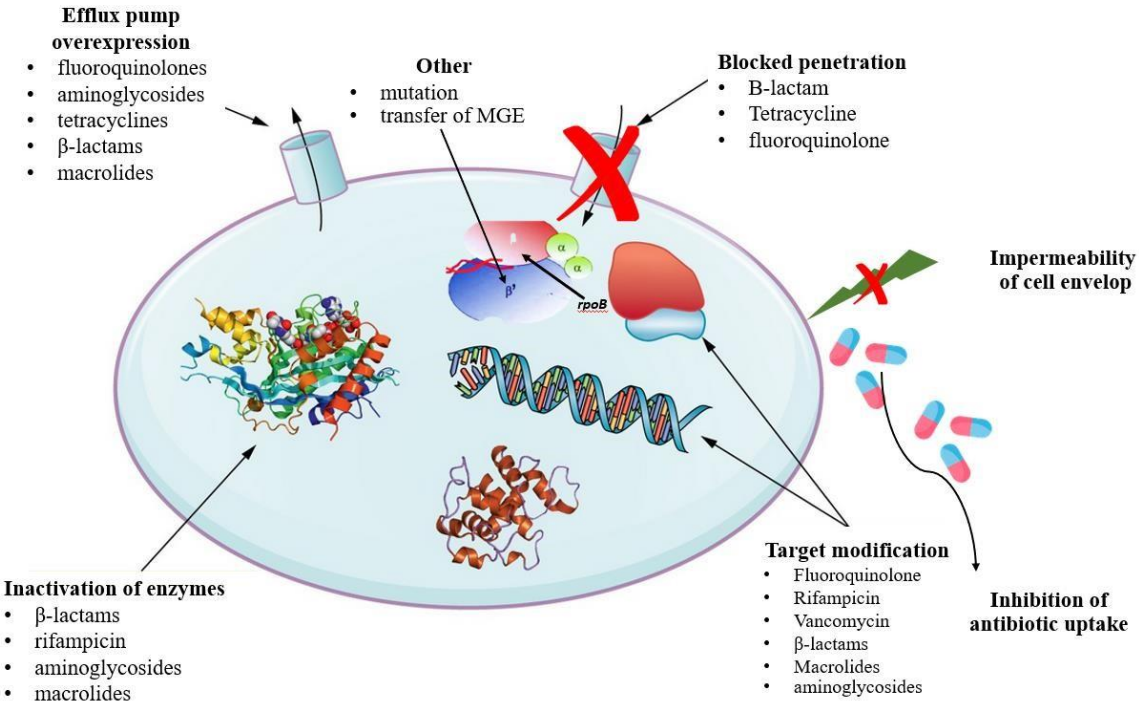


Figure 1: Potential mechanisms of antibiotic resistance; Modified from (https://courses.lumenlearning.com/suny-microbiology/chapter/drug-resistance/).

2.4 Virulence and pathogenicity of *A. baumannii*

The virulence and resistance of *A. baumannii* to different classes of antibiotics have been linked to alterations of some cell wall components such as the LPS, porin proteins, and capsules in the outer membrane. The transport of hydrophobic antibiotics such as chloramphenicol, aminoglycosides, and others including vancomycin and rifampicin is limited by the lipopolysaccharide during diffusion via the outer membrane lipids, while the outer membrane protein (porin) selectively permits the

transport of only small hydrophilic substances such as β-lactams into the periplasmic space, and the capsule acts as a shade against lysing chemicals. An increase in porin expression, upregulation of efflux pump, formation of biofilm, and secretion of hydrolyzing enzymes, have been identified as mechanisms of acquired resistance in Gram-negative bacteria (Magiorakos et al., 2012; Subhadra et al., 2018; Tantisuwanno et al., 2021; Weiner et al., 2016).

2.4.1 Formation of biofilm

Biofilm is a microbial community encased in an exopolysaccharide matrix on surfaces with a high potency of reducing the penetration of antibiotics causing elevated pathogenicity and survival of infectious organisms. This structure has been recognized in promoting and offering protection to *A. baumanni* in the hospital environment both biotic and abiotic and making treatment difficult (Marr, MacDonald, Trivedi, Chakravorty, & Russo, 2020a). Some biofilm-forming *A. baumannii* isolates were detected bearing a polysaccharide matrix which was confirmed absent in non-biofilm-forming isolates (Ebrahimi et al., 2021). Cellular determinants such as biofilm-associated protein (BAP), domain-mediated intracellular signaling molecules, large surface adhesins, and an extracellular polysaccharide matrix were also revealed in isolates of *A. baumannii* (Aliramezani, Douraghi, Hajihasani, Mohammadzadeh, & Rahbar, 2016). A biofilm-forming gene *csgA* was recently identified in isolates of *A. baumannii* conferring resistance to imipenem, meropenem, and doripenem in 66 isolates (Anchana, Girija, Gunasekaran, & Priyadharsini, 2021). Another study has also detected

some biofilm-forming ability in 4 isolates of colistin- and carbapenem-resistance *A. baumannii* conferring resistance to colistin and carbapenem (Ilsan, Lee, Kuo, Lee, & Huang, 2021). More biofilm coding genes were also detected in some *A. baumannii* isolates including the A1S_1507 (fimbrial protein forming gene), A1S_3168 (pilus assembly protein forming), A1S_2042 (transcriptional regulator forming), A1S_0302 (hypothetical protein expressing) and A1S_0114 (an acyl-carrier protein expressing) which is involved in the attachment of biofilm in biotic and abiotic surfaces (Liu, 2022). Recently other genes capable of promoting biofilm formation including the biofilm-associated protein, outer membrane protein A, chaperon-usher pilus, iron uptake mechanism, poly-β-(1, 6)-N-acetyl glucosamine, BfmS/BfmR two-component system, PER-1, quorum sensing (*csuA, csuA/B, csuB, csuC, csuD, csuE, pgaA, pgaB, pgaC, and pgaD adeF, adeG,* and *adeH*) were described in *A. baumannii* isolates (Eze, Chenia, & El Zowalaty, 2018).

Furthermore, the pathogenicity of *A. baumannii* is promoted during quorum sensing. In the biofilm environment bacteria colonies communicate in response to changes in the environment. This communication triggers the production of autoinducers which aid the regulation of bacteria population density and promote the adaptation of cells. The acyl-homoserine lactone (AHL) is commonly identified among *A. baum*annii isolates. AHL is a signaling molecule in both intraspecies and interspecies communication and may aggravate quorum sensing, increase biofilm production, and colonization, and enhance the severity of polymicrobial infection. The role of the *abaR* and interaction with AHL controls the gene expression level of the *abaI* during quorum

sensing and alteration of the genes were currently detected in some isolates of *A. baumannii*.

2.4.2 Overexpression of efflux pumps

Efflux pumps are active transport proteins that prevent the intracellular accumulation of noxious substances in the cell. It is classified into 6 groups or superfamilies which include ATP binding cassette (ABC), small multi-drug resistant family (SMR), multi-drug and toxic compound extrusion (MATE), resistance nodulation division (RND), major facilitator superfamily (MFS) and proteobacterial antimicrobial compound family (PACE) (Du et al., 2018). These proteins can be overexpressed due to mutation, become dysfunctional and impose a high level of resistance on infected cells by the exclusion and reduction of intracellular antibiotic concentration, transporting antibiotics away from the target site such as from cytoplasm to periplasmic space in the case of tigecycline resistance *A. baumannii* (Foong, Wilhelm, Tam, & Pos, 2020). Efflux exclusion of antibiotics can also lead to the exposure of bacteria isolates to sublethal concentration of antibiotics and lesser plasma concentration of antibiotics causes resistance. One of the most important RND efflux pump (AdeABC) consists of three membrane proteins such as AdeC, AdeB, and AdeA which functions as an outer membrane protein, transporter protein, and periplasmic membrane fusion protein respectively. Studies revealed that alterations in the AdeB protein responsible for the transport of molecules within the cytoplasm and phospholipid bilayer are common in *Acinetobacter spp* (Aladel, Abdalsameea, Badwy, Refat, & ElKholy, 2020). The upregulation of RND efflux (AdeABC) which is regulated by a two-component system

AdeRS has been associated with tigecycline resistance due to single amino acid substitution in the AdeRS system. The over-expression of other RND genes coding for AdeIJK, AdeFGH, and AdeABC pumps has been also reported in tigecycline resistance (Sun et al., 2016) and identified in most *A. baumannii* isolates (Kumar, Singhal, Ray, & Gautam, 2020). RND efflux pumps AdeABC and AdeFGH were also identified as biofilm-building pumps (He et al., 2015). More cases of *A baumannii* efflux pump-mediated resistance has been reported in the *abaQ* gene (a member of the MFS transporter) which confers resistance to quinolone (Pérez-Varela, Corral, Aranda, & Barbé, 2018). Mutation of topoisomerase enzymes (DNA gyrase has been noted in the *parC* gene leading to fluoroquinolone resistance of *A. baumannii* (Pérez-Varela et al., 2018). Tetracycline resistance due to efflux pump in *A. baumannii* has been reported with a 2-128-fold reduction in tetracycline MIC in the presence of an efflux pump inhibitor carbonyl cyanide mchlorophenylhydrazone (CCCP) in some isolates bearing tetracycline resistant gene *tetB* (Beheshti et al., 2020). For carbapenem resistance, some efflux pump genes have been identified to be responsible for the increased pathogenicity of *A. baumannii.* The RND efflux pumps were identified as playing a major role in CRAB. The gene encoding AdeABC with transport proteins AdeB and Adeijk were over-expressed and upregulated respectively (Beheshti et al., 2020). Meropenem and imipenem resistance were associated with the AdeABC efflux system (Beheshti et al., 2020). Another study has established that *adeB* and *adej* were the most common efflux pump genes in CRAB (Hasani et al., 2021). Recently, almost 100% *A. baumannii* isolates obtained from southeast Asia were found with many efflux pump coding genes

such as (*adeN, adeR, adeS, adeABC*) of the RND class with an increased expression of other pumps of the MFS, SMR, and MATE superfamily (Wareth et al., 2021).

2.4.3 Modification of cell wall components and capsular polysaccharides

The complexity of the cell wall of *A. baumannii* plays a key role in promoting resistance due to the lipopolysaccharide-imposed barrier. LPS holds the lipid-A (an immuno-stimulator) responsible for the signal transduction between the pathogen and the host immune system through the toll-like receptor 4 (TLR-4). Recent research has identified a mutant type *A. baumannii* that lowers TLR-4 signaling and could survive in the host cell without lipid A expression (Monem et al., 2020). The inability of the species to express this component prevents signal transduction hence a reduced immune response or causes total absence (Mat Rahim, Lee, Strych, & Abubakar, 2021).In addition, the release of some cytotoxic materials and evasion of the host immune response in *A. baumannii* which triggers the host inflammatory response has been connected with the LPS (Tiku & Tan, 2021). The overexpression and mutation in the gene coding for the LPS such as *lpsB, lpxA, lpxB, lpxC, lpxD, lpxL*, and *lpxM* are the major causes LPS associated antibiotic resistance recognized in *A. baumannii* (Chukamnerd et al., 2022b). Antibiotic resistance conferred by the lipopolysaccharide may originate due to the under-expression of LPS, loss of LPS, and the less production of cofactors involved in the synthesis of LPS. In addition, the protective activity of capsular polysaccharides against the host innate immune system (complement) and the

phagocytic cell have been recognized in *A. baumannii* as another pathogenetic feature in both *in vivo* and *in vitro* studies (Beheshti et al., 2020)

2.4.4 Loss or modification of porin

Apart from the transportation limitations on hydrophobic antibiotics, the reduction of outer membrane porin in *A. baumannii* has been identified as the cause of decreased permeability of some hydrophilic antibiotics such as carbapenems. Porin protein permits the passage and transportation of antibiotics and loss, or modification of the protein structure limit the influx of substances into the cell. Porin exclusion of antibiotics is another reason for the lack of active treatment options for the control of CRAB. However, the CarO (carbapenem-associated outer membrane protein) and OprD are specialized porins responsible for the influx of carbapenems antibiotic, mal regulation in the gene that codes for these porins have been detected mediating resistance to carbapenems (Gopikrishnan & Doss, 2023).

2.4.5 Enzymatic degradation of antibiotics

Hydrolytic enzymes capable of destroying functional groups of antibiotics are synthesized by bacteria leading to the degradation of the agent due to lack of binding site as in the breakdown of the β-lactam ring. Several carbapenemases have been identified in *A. baumnnii* conferring resistance to imipenem, meropenem, and doripenem. Mostly among CRAB isolates the oxacilinases have been detected such as the $OXA_{23,}$ OXA_{40}, OXA_{51} $OXA_{58,}$ and OXA_{143} . The OXA_{58} which is mostly coded by

plasmid is been disseminated all over the world (Kumar et al., 2019). Other carbapenemases mediating resistance include the NDM-1, VIM-1, SIM-1, and IMP of the class B β-lactamases as indicated in Table 1. Studies have also revealed the presence of aminoglycoside-modifying enzymes that may mediate resistance to aminoglycosides (Wareth et al., 2021).

Table 1. Some clinically relevant carbapenemase identified in *A. baumannii* (Chukamnerd et al., 2022b)

Carbapenemases		
Class A	**Class B**	**Class C**
Klebsiella pneumonia carbapenemases (KPC), Guiana extended spectrum βlactamases (GES)	Imipenemase (IMP), Verona-integron-encoded metallo-β-lactamases (VIM), Seoul imipenemase (SIM), New Delhi metallo-βlactamases (NDM)	Oxacilinease (OXA): OXA51-like group, OXA23-like group, OXA40/24-like group, OXA58-like group, OXA143-like group, OXA148-like group

2.4.6 Mobile genetic elements (MGEs)

2.4.6.1 Transposons (insertion sequence), plasmids, and integrons (gene cassette)

Gene mutation is another instance that has been associated with *A. baumannii* resistance to many antibiotics because it has massive resistance islands and the ability to easily acquire resistance from other bacteria species (Kumar et al., 2019) A previous study has disclosed that *A. baumannii* can develop resistance to antibiotics

while treatment is going on and is facilitated by mobile genetic element (MGE) (Monogue, Sakoulas, Nizet, & Nicolau, 2018). Resistance mediated by MGE manifests in bacteria phenotypes in the form of modification, overexpression, and alteration. Insertion sequence is a small DNA fragment that comprises about 2500 bp and has been reported with the ability to alter bacteria genome. *ISAbal* is an insertion sequence identified in isolates of *A. baumannii* and has been associated with the activation of OXA_{51} carbapenemase. Integrons incorporate resistance genes and promote transcription and expression. Carbapenem resistance in *A. baumannii* has also been linked to integron-carrying genes responsible for the synthesis of bla_{VIM}, and bla_{SIM} carbapenemases (Nie et al., 2020). Some regions of the bacteria genome with diverse antibiotic resistance mediating genes were identified in *A. baumannii* known as resistance islands. Another research evinced that about 86 kbp resistance islands with a cluster of 45 antibiotic resistance genes were also present in some *A. baumannii* strains. However, in Asia, the $bla_{23\text{-like}}$ genes were detected in CRAB isolates carrying the AbaR4-type resistance island (Guo, Xun, & Han, 2018). In addition, circular DNA (plasmids) deposited during conjugation confer antibiotic resistance. Plasmid-mediated resistance in *A. baumannii* has been reported especially in CRAB isolate transferring resistance gene coding for OXA_{58}, OXA_{24}-like carbapenemases (Tiku & Tan, 2021).

Table 2. Some genes conferring resistance to carbapenems and rifampicin in *Acinetobacter*

S/N	Mode of resistance	Genes	Functions	Antibiotics	Reference

1.	Loss or modification of Porin	*carO, oprD*	CarO and OprD are specialized porins. responsible for the influx of carbapenems antibiotic	Carbapenems	(Tiku et al., 2021)
2.	Overexpression of efflux pumps	*adeB, adeJ, adeN, deR, adeS, adeA, adeC*	Regulate the extrusion of substances from the cell	Carbapenems	(Katsube, Echols, & Wajima, 2019)
3.	Modification of penicillin-binding protein and hydrolysis by carbapenemases	*pbpG*	Regulate synthesis of modifying enzymes	Carbapenems	(Chukamnerd et al., 2022b)
4.	Chromosomal mutation in RNA polymerase (RNAP) β-subunit of the rpoB target gene and a missense mutation in rpoB	*rpoB*	Codes for the beta subunit of the RNA polymerase which presence binding site for rifampicin	Rifampicin	(Robin et al., 2022)
5.	Enzymatic modification of rifampicin by ADP-ribosyl-transferase ARR-2	*arr-2*	Plasmid-mediated resistance gene	Rifampicin	(Cai et al., 2017b)
6.	Biofilm formation	*bfmR, csgA*	Code and regulate the csu/BABCDE chaperon-ursher the system involves in the formation and attachment of pilli.	Carbapenem	(Wong et al., 2017)
7.	Lipopolysaccharide loss or alteration membrane permeability of the Drug	*lpx*	Permit passive diffusion of rifampicin	Rifampicin	(Robin et al., 2022)

Collectively, the virulent factors below contribute to the pathogenicity of *A. baumannii* which begins from immune evasion, adherence, biofilm formation, enzymatic activation, iron uptake, siderophore formation, misregulation, and serum resistance.

2.5 Pathogenicity of *Acinetobacter baumannii*

2.5.1 Immune evasion

A. baumannii adheres to the epithelial cells of the respiratory tract through the outer membrane protein A (OmpA). It has been noted that other cellular components such as the mitochondria and the nucleus are invaded by the ompA leading to the release and expression of the proapoptotic molecule cytochrome c and the apoptotic-inducing factor which trigger cell death (Guo et al., 2018).

2.5.2 Serum resistance

This occurs due to the neutralization of the factor H which controls the alternative complement pathway-mediated killing. The bypass of the pathway leads to the rapid differentiation of the CD4 cell, activation and maturation of the dendritic cells, and premature apoptosis of the cells. In a previous study, *A. baumannii* isolates were identified with a high survival rate in human serum and about 4 isolates survived amidst the cell components of human whole blood (Colquhoun & Rather, 2020). The penicillin-binding protein and the associated gene *pbpG* have been linked to bacterial cell stability contributing to the pathogenicity of *A. baumannii* (Monogue et al., 2018).

2.5.3 Outer membrane proteins (OMP) and vesicle (OMV)

The role of the *A. baumannii* OmpA in the disease pathogenetic pathway has been described (Nie et al., 2020). Currently, it was demonstrated that onset of disease conditions, the ompA facilitates the adherence process, host cell invasion, serum resistance, and epithelial cell invasion and induces apoptosis (Guo et al., 2018). Research has also associated the pathogenicity of *A. baumannii* with several defective proteins such as catalase, phospholipases, proteases, superoxide dismutase, and degradative enzymes harbored by an outer membrane vesicle. In addition, these proteins have been detected in infection sites and found to be responsible for the increase in the innate immune response which leads to tissue damage (Tiku et al., 2021).

2.5.4 Iron uptake

Siderospores are tiny iron-chelating secretions that bind to ferric ions and enables *A.baumannii* to capture iron under iron shortage condition (Katsube et al., 2019). The secretion is often conveyed by a tripartite efflux pump (Robin et al., 2022). Usually, *A. baumannii* cannot obtain iron via transferrin or lactoferrin, they help acquire and accumulate iron. Despite the considerable synthesis of iron, the availability of physiologically active ferric iron is constrained by the lower solubility of iron in an aerobic environment and chelation by substances like hemoglobin and the ferric binding protein transferrin.

2.5.5 Regulation

The BfmRS is a regulatory system encoded by the genes *bfmR* and *bmfS* which are responsible for the activation of the Usher-chaperone assembly system (CsuA/BABCDE) involved in the production of the pili needed for biofilm formation on polystyrene surfaces (Saipriya, Swathi, Ratnakar, & Sritharan, 2020). The deactivation of the BfmS may affect the regulatory process and translational modification of OmpA leading to the loss of OmpA-mediated pathogenesis.

2.5.6 Enzymes

A. baumannii synthesized phospholipases are lipolytic enzymes that often cleave with the host cell membrane phospholipases and trigger lysis of the host cell. The production of the phospholipase is controlled by the gene *plC* and *plcD.* The phospholipases have been described with the ability to hydrolyze human erythrocytes, aid iron acquisition, invasion of epithelial cells, and serum resistance (Morris, Dexter, Kostoulias, Uddin, & Peleg, 2019). The expression level of the genes may also be connected with the pathogenicity of *A. baumannii.*

2.6 Adjunctive antibiotics and mechanisms of action

2.6.1 Carbapenems

Carbapenem is a class of beta-lactam antibiotics with the highest level of stability against βlactamases. It is a last resort antibiotic administered for the treatment of Gram-negative bacteria and the choice treatment option for patients with severe infections of extended-spectrum beta-lactamases producing Enterobacteriaceae besides *E*

coli (Saipriya et al., 2020) Carbapenem includes imipenem cilastin, meropenem, ertapenem, doripenem, panipenem-betamipron and biapenem. Imipenem and meropenem are active against MDR organisms at a higher dose. This class of antibiotics is not administered orally due to PH-related instability in the gastro- intestinal tract, poor lipophilicity to cross intestinal epithelium, and exclusion by efflux pump present on the surface of enterocytes (Morris et al., 2019) In absence of carbapenems, cefepime, aminoglycosides, fosfomycin, temocillin, piperacillin-tazobactam, and ceftazidime-avibactam are given (Vos et al., 2011). Carbapenems act against Gram-negative bacteria by penetrating the periplasmic space and binding to penicillin-binding proteins (PBPs) which are involved in the synthesis of cell wall inhibiting its production thereby causing lyses perforation, and death of the cell due to osmotic instability.

CRAB was first reported in 1991 with a significant rise in the number of resistant Phenotypes (MarÝ-Almirall et al., 2017). From then onward several strains have emerged conferring resistance to various types of carbapenem antibiotics globally. However, a large proportion of antibiotic resistant can be linked to CRAB with an increase in the percentage of occurrence of almost 58% recorded in North American hospitals and 94.5% collated in Greece. (MarÝ-Almirall et al., 2017) Since the identification of carbapenem-resistant Acinetobacter *baumannii,* there have been no new antibiotics with the potency to treat carbapenem-resistant *A. baumannii* except for tigecycline that has been associated with increased mortality, low blood level and toxicity and excessive death (Hasan et al., 2014).

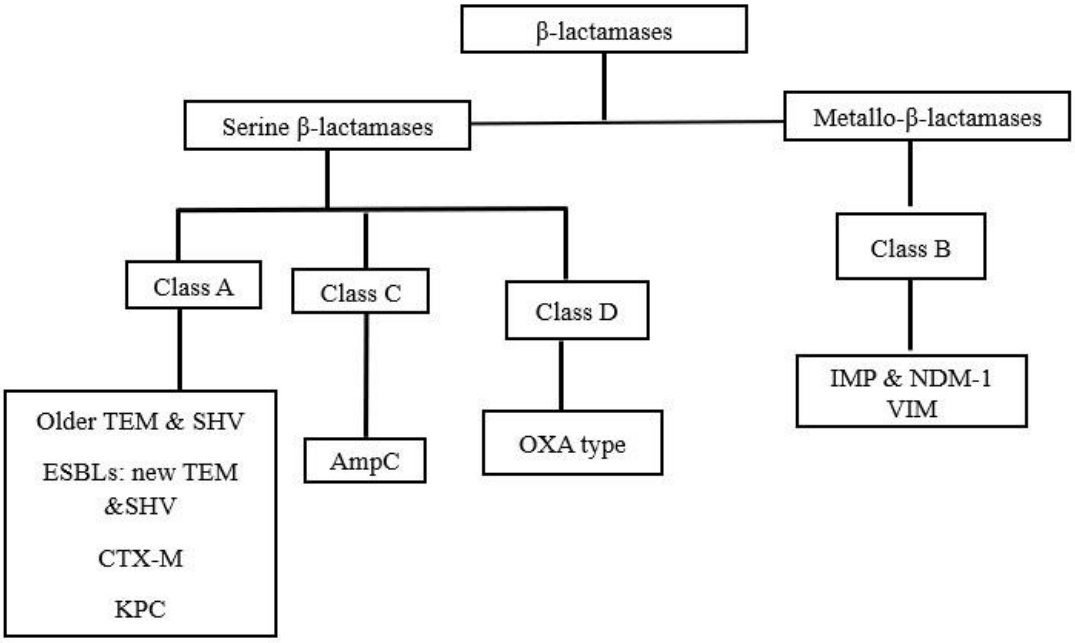


Figure 2. The Ambler classification of β-lactamases

The above classification of β-lactamases is based on the primary protein structure of the enzymes. The active site of the enzymes of class A, C, and D enzymes contains serine residue which is involved in the hydrolysis of the β-lactam ring. The active site of class B enzymes contains zinc ions as a cofactor that enhances the function of the enzyme and is called metallo-β-lactamases. ESBL: extended spectrum β-lactamases; (TEM, Temoniera; SHV, sulfhydryl variable; CTX-M, cefotaxime β-lactamase;) KPC, *Klebsiella pneumoniae* carbapenemase; OXA, oxacillinase; IMP, imipenemase type carbapenemase; NDM-1, New Delhi metallo-βlactamases; VIM, verona integron-encoded metallo-β-lactamases. AmpC β-lactamases

2.6.2 Rifampicin

Rifampicin is a bactericidal drug obtained from nature or synthesized by a bacterium called *Amycolatopsis rifamycinica*. It belongs to a family of antibiotics known as ansamycins. Rifampicin is also a drug metabolizer inducer and a P-glycoprotein capable of breaking down other chemotherapeutic substances and as such is not combined with medications like anticonvulsants (phenytoin, anticoagulants (warfarin, dabigatran), dapsone, chloramphenicol, clarithromycin, antiretroviral drugs (eg zidovudine), oral contraceptives, (Petrova & Petrov, 2021). It is commonly used for the treatment of tuberculosis, mycobacterium infections, legionnaires' disease, and leprosy (Pfaller & Diekema, 2012). Some derivative of these drugs includes rifabutin, rifapentine, rifalazil, and rifaximin. Rifampicin has a high affinity to bind to the RNA of prokaryotic cell and always act by cleaving to the β-subunit of the RNA polymerase enzyme and halting transcription and translation (Singkham-In & Chatsuwan, 2018).

Rifampicin can be administered orally or intravenously and requires the measurement of liver enzymes and blood count (Aliramezani et al., 2016) However, patients are advised to take a periodic liver functioning test to detect any advert effect of this drug on the liver cells. This medication has been found effective in combination with other medications. Indications from previous research have commended the use of rifampicin as an active substance against varieties of pathogenic organisms (Bonapace, Bosso, Friedrich, & White, 2002). Rifampicin has been used as an active chemotherapeutic compound for the treatment of microbial infections (Singkham-In & Chatsuwan, 2022). A synergistic result has been obtained in *A. baumannii* infected

murine between rifampicin and Polymyxin B derivative and has passed the phase 1 clinical trial (Singh et al., 2017). The utilization of rifampicin as prophylaxis in a disease condition is shown in the case of *Neisseria meningitides* (meningococcal) infections. More information on this subject has pointed out that rifampicin may be used in the treatment of β-lactam resistance *Streptococcus pneumoniae* infection and for the control of invasive *Haemophilus influenza* (Bonapace et al., 2002). The antibacterial susceptibility of this medication on clinically relevant organisms indicates a minimal inhibitory concentration (MIC) of 0.002 – 64 µg/mL for *Mycobacterium tuberculosis*, 0.125 µg/mL for *Mycobacterium bovis*, ≤ 0.006 – 256 µg/mL for methicillin-resistant *Staphylococcus aureus* (MRSA), and 0.005 µg/mL for *Chlamydia pneumoniae* (Bardbari et al., 2017). Rifampicin has also been used as an adjunctive therapy for the treatment of resistance GNB such as the CRAB as in Table 3.

Table 3. Rifampicin combination therapy with some commonly used antibiotics.

Type of study	RIF (MIC)	RIF in combination (MIC)	FICI	Antibiotics	References
In vitro	4-128 µg/mL	4 4		Imipenem Sulbactam	(Bai et al., 2015)
In vitro	1-16 µg/mL	0.0625-32 0.0625-0.5 0.125 -1	-	Biapenem Colistin Tigecycline	(Shen et al., 2020)
In vitro	4-32 µg/mL	2-16		Cefoperazone-Sulbactam	(Lim et al., 2019)
In vitro	0.5-2 µg/mL	-	0.25-1.25	Meropenem	(Čiginskienė et al., 2020)
In vitro	1-64 µg/mL	-	0.25-0.51	Colistin	(Wong et al., 2017)
In vitro	4-128 µg/mL		0.31-1.5	Sulbactam	(Marr, MacDonald,

					Trivedi, Chakravorty, & Russo, 2020b)
In vitro	2-16 μg/mL	0.25-8		Imipenem	(Aliramezani et al., 2016)
In vitro	2-64 mg/dL	0.28-0.56 0.28-1.0(μg/L)		Imipenem Meropenem	(Teo et al., 2015)
In vitro	1-4 μg/mL		0.19-0.56	Polymyxin B	(Ilsan et al., 2021)
In vitro and in vivo	4-128 μg/mL	<0.25-16	0.09-0.38	Lysine	(Rumbo-Feal, 2018)
In vitro	0.5-16 (mcg/ml)		0.25-0.75	Colistin	(Hasan et al., 2014)

However various mechanisms of Rifampicin resistance have been recognized in *A. baumannii* isolates which include the modification of resistance determining region of the rpoB, efflux pump exclusion of antibiotics due to overexpression of efflux pump genes, plasmid-mediated enzymatic modification of rifampicin by ADP-ribosyl transferases Arr-2 and low intake of rifampicin (Cai et al., 2017b).

Table 4. Mode of action of some commonly used antibiotics

S/N	Classes of antibiotics/examples	Mode of action	References
1.	β-lactams Carbapenem: imipenem, doripenem, meropenem, ertapenem Cephalosporin: cephalexine, cefaclor, cefoperazone, ceftazidime, cefepime, ceftaroline Penicillin: ampicillin, nafcillin, ticarcillin, methicillin, carbenicillin Monobactam: aztreonam	Cell wall inhibition	
2	Aminoglycosides amikacin, gentamicin, tobramycin, neomycin, streptomycin	Inhibits protein	(Badmasti, Siadat, Bouzari,

		synthesis.	Ajdary, & Shahcheraghi, 2015)
3	oxazolidonones linezolid, tidezolid		
4	Tetracyclines minocycline, tetracycline, tigecycline, democlocycline, doxycycline,		
5	Streptogramins quinupristin, chloramphenicol		
6	Macrolides azithromycin, clarithromycin erythromycin,		(Badmasti, Siadat, Bouzari, Ajdary, & Shahcheraghi, 2015)
7	Quinolones/fluoroquinolones Nalidixin acid /ciprofloxacin, sparfloxacin, levofloxacin, norfloxacin	Inhibit DNA replication	
8	Rifampicin	Inhibit RNA transcription	
9	Sulfonamides Sulfamethoxazole, sulfasalazine, sulfisoxazole,	Inhibits folate synthesis	
10	Glycopeptides Vancomycin, telavancin	Inhibit cell wall synthesis	
11	Other Polymyxin, colistin, daptomycin		

With the recent emergence of mutant organisms and the failure of monotherapy in the treatment of CRAB, other methods have been employed to increase the efficacy of chemotherapeutic agents for the provision of beneficial and personalized treatment. The methods include the use of natural products, plant extracts and phytochemicals, microbial enzymes and metabolites, whole microbial cells (lactic acid bacteria; bacteriocin), Use of bacteriophages (phage therapy), and antimicrobial combinations therapy. In this research, rifampicin will be used as the primary antibiotic in combination with imipenem, meropenem, doripenem, tigecycline, minocycline

ciprofloxacin, levofloxacin, trimethoprim-sulphamethoxazole, amikacin, gentamycin, tobramycin, fosfomycin, colistin, polymyxin B, and chloramphenicol.

2.7 Recent treatment options for CRAB

- Cationic peptides such as polymyxins can be administered as a membrane permeabilizer.
- Concomitant administration of inhaled polymyxin and aminoglycosides in VAP.
- Combination therapy with inactive antibiotics such as carbapenem, old aminoglycosides, tigecycline, fosfomycin, rifampicin, and novel agents (plaszomicin, eravacycline) (Bardbari et al., 2018).
- The use of cefiderol (Lucia et al., 2022)

2.8 Combination therapy

Combination therapy is a strategy employed to enhance the efficacy of antimicrobial agents by combining more than one active substance for the treatment and management of disease conditions.

2.9 Benefits of combination therapy

Combination therapy involves multi-site targeting using a different mechanism of action to mediate bactericidal response against MDR organisms. It broadens the antibacterial spectrum, reduces the emergence of resistance, enhances the

efficacy of antibiotics, and is convenient for the treatment of polymicrobial infections, limiting of occurrence of hetero resistance among the subpopulation of bacteria isolate.

CHAPTER 3

MATERIALS AND METHOD

3.1 Chemicals and media

Antibiotics including ciprofloxacin, levofloxacin, imipenem, and meropenem were procured from Siam Bheasach Co., Ltd, Bangkok, Thailand, and doripenem was supplied by Shionogi pharma co., Ltd Kanegasaki plant, Iwate, Japan. Fosfomycin was obtained from Meiji Seikakaisna, Ltd. Tokyo, Japan. Amikacin, gentamycin, tobramycin, trimethoprim/sulfamethoxazole, chloramphenicol, and carbonyl cyanide m-chlorophenyl hydrazone were purchased from Sigma-Aldrich, (Saint Louis, MO, USA). Tigecycline and minocycline were obtained from Pfizer Inc. Philadelphia, PA, USA. Colistin was obtained from Sigma-Aldrich, Co. 3050 Spruce Street, St Louis, Mo 63103 China. In addition, polymyxin B was supplied by MedChem, express USA. Rifampicin, Tryptic soy agar and broth (TSA and TSB), and cation-adjusted Mueller Hinton broth II (CAMB-II) were supplied by HiMedia Laboratories Pvt. Ltd, Mumbai, India. Other culture media including Mueller Hinton agar and broth were supplied by Becton Dickinson & Co. Difco, Franklin Lakes, NJ, USA. Resazurin, MTT, and Crystal violet dye were supplied by Pfizer Inc. Philadelphia, PA, USA. The TIANamp bacteria DNA extraction kit used in this study was procured from Tiangen, Beijing, China.

3.2 Collection of *Acinetobacter baumannii* clinical isolates

Carbapenem-resistant *A. baumannii* (CRAB) (n = 218) clinical isolates collected from patients admitted in hospitals of Southern Thailand (Songkhla, Pattani, Phatthalung, Trang, Satun) were enrolled for the study. CRAB isolates were previously characterized as Gram-negative, oxidase-negative, nonmotile, non-fermenting coccobacilli using standard biochemical tests (Vos et al., 2011) and by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) (MarÝ-Almirall et al., 2017). Bacteria isolates were picked from the −80°C storage facility and subcultured in tryptic soy agar (TSA) using the streak plate method.

3.3 Screening for rifampicin resistance

Rifampicin sensitivity test was conducted using disc diffusion assay and rifampicin resistance isolates of CRAB were identified as previously described with slight modification (Petrova & Petrov, 2021). Since rifampicin is not indicated for the treatment of *A. baumannii*, the CLSI, 2020 guideline for disc diffusion breakpoint for rifampicin against *Staphylococcus spp* (susceptible; ≥20, Intermediate; 17-19 and resistant; ≤ 16) was employed. Briefly, a colony of each isolate was inoculated into a sterile micro-centrifuge tube containing Mueller Hinton broth and cultured to log phase for (3-5h). The culture was converted to MacFarland's, adjusted culture was evenly spread on the plate of Mueller Hinton agar. Rifampicin discs of 5 µg were properly stationed on each of the plates. All plates were then incubated at 37°C for about 16 to 18 h.

3.4 Antibiogram of carbapenem-resistant isolates

CRAB isolates that were resistant to rifampicin were further used for the study and were treated with 15 commonly used antibiotics including carbapenem (imipenem, meropenem, and doripenem), aminoglycosides (amikacin, gentamycin, and tobramycin), glycylcyclines (minocycline and tigecycline), fluoroquinolone (ciprofloxacin and levofloxacin), trimethoprim-sulphomethoxazole, fosfomycin colistin, polymyxin B, trimethoprim-sulfamethoxazole, and rifampicin in monotherapy. Broth mico-dilution assays were used to assess the minimum inhibitory concentrations (MIC) of the antibiotics on rifampicin- and carbapenem-resistant isolates as detailed (Pfaller & Diekema, 2012). Briefly, serial two-fold dilutions of antibiotics were prepared in cation-adjusted Mueller–Hinton II broth. Aliquots (100 µL) of the diluted bacterial suspension (1×10^6 CFU/mL) will be exposed to 100 µL of varying antibiotic concentrations and incubated at 37 °C for 18 h. Ten microlitter of resazurin was then added and placed in the incubator for 2h for clarity of the result. MIC will be expressed as the lowest concentration of the antibiotic without microbial growth.

3.5. Antimicrobial combination assay

The antibacterial activity of a combination of rifampicin with other antibiotics including amikacin, gentamycin, tobramycin, levofloxacin, ciprofloxacin, imipenem, meropenem, doripenem, fosfomycin, trimethoprim-sulfamethoxazole, chloramphenicol, colistin, polymyxin B tigecycline, and minocycline was investigated by checkerboard test as detailed with slight modifications (Singkham-In & Chatsuwan,

2018). Serial 2-fold dilutions of rifampicin were prepared in Mueller-Hinton broth II in a 96-well microtiter plate down the rows while an equal volume of colistin was 2-fold serially diluted on falcon tubes and mixed in appropriate wells in the column. Then 100 µL aliquot of bacteria culture with a final concentration of about 5×10^5 CFU/mL and an equal volume of both antibiotics' mixture was placed in each of the wells. A row and column with colistin and rifampicin alone were used to mark the MIC of each antibiotic respectively while wells containing a 5×10^5 CFU/mL bacteria culture alone were utilized as control. Plates were then incubated for 18 h at 37°C. The fractional inhibitory concentration index (FICI) was calculated according to the equation FICI = FICa + FICb = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). The FICI results for each combination were interpreted as follows: FICI ≤ 0.5, synergism; 0.5 < FICI < 1, additive; 1 ≤ FICI < 2, indifference; FICI ≥ 2, antagonism (Bonapace et al., 2002).

$$\text{FICI} = \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}}$$

3.6 Time-kill assay

Time-dependent killing dynamics of some representative isolates were conducted as previously described with modification (Leelasupasri, Santimaleeworagun, & Jitwasinkul, 2018) using rifampicin alone and in combination with ciprofloxacin, trimethoprim/sulfamethoxazole, minocycline, tigecycline, colistin, meropenem, doripenem, and imipenem. In brief, overnight culture was adjusted 10^6CFU/ml from 0.5 McFarland and treated with 1/2 rifampicin + 1/2 colistin, 1/2 rifampicin + 1/4 colistin,

1/4 rifampicin + 1/4 colistin, 1/4 rifampicin + 1/8 colistin. Treated bacteria cultures were then monitored for 24 h at intervals of 2, 4, 8, 12, and 24 h. At each time limit, a serial 10-fold dilution in normal saline of each bacteria culture was performed and the drop plate method was used to enumerate bacteria colonies after incubation at 37 C for 18 h. All experiments were repeated twice independently. Synergism was defined as a 2-log reduction in CFU/mL when compared with the most active single antibiotic treatment, whereas bactericidal activity was defined as a ≥3-log reduction in CFU/mL when compared with the number of viable cells at time zero (0 h) and bacteriostatic at 2 log reduction in CFU/ml.

3.7 Efflux pump detection assay

The phenotypic detection of the rifampicin-resistant efflux pump was performed in the presence of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) as previously described with modifications (Singkham-In & Chatsuwan, 2022). CRAB isolates that were resistant to rifampicin at MIC ≥ 16µg/mL were cultured to log phase for 4h. Then a serial 2-fold dilution was conducted with a 50µL aliquot of rifampicin and 50µL of 20µg/ml was placed in each well. One hundred (100µl) aliquot of adjusted bacteria culture at 10^6 CFU/mL was included in the wells. The plates were incubated for 18h and the minimal inhibitory concentrations of rifampicin against CRAB isolates in the presence of CCCP were taken. The positive phenotype of overexpression of the efflux pump was defined as at least a 4-fol reduction of rifampicin MIC observed in the presence of CCCP.

3.8 Biofilm formation assay

Crystal violet assay was conducted to identify biofilm-forming isolates among CRAB clinical isolates in 96 well microtiter plates (Singh et al., 2017) with slight modifications. Briefly, isolates were sub-cultured on TSA for 24 h and a colony of each was inoculated into a sterile tube and grown overnight in MHB. The culture was adjusted to 10^6 CFU/mL and resuspended in TSB. A 200 µL aliquot of each bacteria suspension was seeded in a 96-well microtiter plate and incubated for 24 h at 37˚C. Planktonic suspensions were aspirated, and the wells were washed twice with 300 µL of sterile phosphate-buffered saline (PBS). Plates were drained completely and allowed to dry in the laminar hood for 1 h. Completely dried wells were stained with 200 µL of 0.1% of crystal violet for 30 min. After staining, the excess crystal violet was aspirated, and the wells were washed with distilled water to remove residual crystal violet dye. The plates were again dried in the incubator until the wells were completely dried. The crystal violet absorbed in biofilm biomass was solubilized in 200 µL of DMSO, and the absorbance was measured at an OD of 595 nm using a multimode plate reader EnSpire. Blank wells with media alone were maintained as negative control and all experiments were done in triplicates for three independent repeats. The isolates were classified into four groups based on the ability to form biofilm following the interpretation criteria as below: ODcut = ODavg of negative control + SD of OD of negative control OD ≤ ODcut = non-biofilm formers ODcut < OD ≤ 2 × ODcut = weak biofilm formers, 2 × ODcut < OD ≤ 4 × ODcut = Moderate biofilm former, OD > 4 × ODcut = Strong biofilm former (Bardbari et al., 2017).

3.9 Viability of biofilm cells

The MTT (3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was employed to monitor the effects of single antibiotics treatment on the viability of biofilm cells (Badmasti et al., 2015). Briefly, 1 mL of 10^6CFU/mL of bacterial suspension in TSB was seeded in a 24-well microtiter plate and incubated at 37˚C for 96 h. Fresh media were added at intervals to ensure that the wells were not dried. After the incubation period, unattached cells were aspirated, and plates were carefully washed with PBS without disruption of the sessile cells. Wells were treated with 16, 8, and 4 MICs of respective antibiotics, and the plates were then incubated for 24 h at 37°C. Untreated wells with bacteria alone were maintained as a control. Antibiotics were removed and the plates were incubated with 300µl of 0.05% MTT dye for 2 h. MTT-treated wells were then washed, and dried before solubilizing with DMSO. Absorbance was taken at OD595, and all experiments were performed in triplicate for three independent repeats.

Adjunctive antibacterial therapy of rifampicin and carbapenems on 96 h established biofilm was also investigated. The 96 h established biofilms were treated with 500 µL of imipenem, meropenem, and doripenem in combination with 500 µL of rifampicin at various concentrations, including 8 MIC + 4 MIC, 8 MIC + 2 MIC, 4 MIC + 4 MIC, and 4 MIC + 2 MIC, respectively. Staining with MTT dye was done accordingly. Experiments were conducted in duplicate and repeated thrice. All results

were presented as: Percentage (%) biofilm inhibition = $\frac{Average\ mean\ of\ treated\ wells}{Average\ mean\ of\ untreated\ wells} \times 100$

3.10 Scanning electron microscopy (SEM)

The effects of antibiotic treatments on bacteria cells membrane were investigated using a scanning electron microscope as previously described with modifications (Remuzgo-Martínez et al., 2015). In brief, overnight bacteria suspension was adjusted to 10^6 CFU/mL and treated with several concentrations of antibiotic monotherapy at MICs of individual antibiotics and in combinations at MIC + 1/2 MIC, 1/2 MIC + 1/2 MIC, and 1/2 MIC+ 1/4 MIC of rifampicin and meropenem, respectively. Untreated bacterial cultures were used as a control. All tubes were further incubated at 37˚C for 3 h with constant agitation at 150 rpm. Cells were then harvested at 8,000 rpm for 5 min and resuspended in PBS. Briefly, 100 µl of 108 CFU/mL of washed bacterial cells was fixed on a glass slide using 3% glutaraldehyde solution for 2 h. Cells were dehydrated with concentration gradient ethanol (20, 40, 60, 80, and 100%) at an interval of 15 min. Samples were dried and gold coated before microscopy.

3.11 Genome DNA extraction, library preparation, and sequencing

The WGS of 20 CRAB isolates were obtained from the previous study (Chukamnerd et al., 2022), and 10 isolates without genomic data were sequenced in this study. In brief, *A. baumannii* clinical isolates were grown on a TSA overnight then a single colony of each bacteria isolates was inoculated in a sterile tube containing LB

media and incubated at 37°C for 24 h. Genomic DNA extraction of the isolates including SK024, SK052, SK065, ST002, SK068, TR125, TR131, TR009, and ST004 was performed using the TIANamp Bacteria DNA kit (Tiangen, Beijing, China), based on the manufacturer's instruction. All extracted DNA samples were preserved and further analyzed at the Beijing Genomics Institute (BGI) in China. The integrity check of all extracts was conducted and scored based on purity and concentration using the Agarose Gel electrophoresis and Qubit Fluorometer (Invitrogen) respectively. The qualified DNA samples were sequenced with the MGISEQ-2000 platform with 150-bp pair-end reads.

3.12 Genome assembly and annotation

A *de novo* assembly was further conducted on 10 isolates including the SK024, TR009, ST004, SK 065, SK052, ST002, TR125, TR131, ST011, and SK068 using SPAdes v3.12 (Bankevich et al., 2012). Then the Quast v5.0.2 and Busco v5.1.2 were utilized to assess the quality and completeness of the bacteria genomes, respectively (Park et al., 2016; Seppey et al., 2019). Genomic annotation of the assembled genomes of all isolates was performed using Prokka v1.12 (Seemann et al.,2014).

3.13 Whole-genome sequence (WGS) analysis

The WGS of all CRAB isolates was used for the identification of mechanisms of antibiotic resistance in the isolates. The multilocus sequence typing (MLST), acquired AMR genes, and plasmid types were identified using streamer v0.7.2 with databases of ResFinder (Zankari E, Hasman H, Cosentino S et al., 2012) and

PlasmidFinder (Carattoli A, Zankari E, García-Fernández A et al.,2014) in the center for genomic epidemiology (CGE) (http://www.genomicepidemiology.org/) and the MLST in PubMLST (https://github.com/tseemann/mlst) (Jolley KA, Bray JE, and Maiden MC et al., 2018). Virulence-associated genes were also searched using blastn with the virulence factor database (VFDB) (http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Acinetobacter) (Chen L, Yang J, Yu J et al., 2005). Chromosomal mutations in the *rpoB* gene of the bacteria genomes were investigated by aligning the reference sequence of the *rpoB* gene from the *A. baumannii* ATCC 19606 genome (NZ_CP015121.1) using Geneious prime® software. The effects of each variant were further analyzed using the Mutpred 2® software (http://mutpred.mutdb.org). Furthermore, a study was conducted to assess the diversity of resistance and the spread of AMR genes. A comparative analysis of the genomic data of CRAB clinical isolates from this study and previous WGS results from Chukamnerd et al. (2022) (Chukamnerd A, Singkhamanan K, Chongsuvivatwong V et al., 2022) was conducted using Roary software® v3.13.0 (Page AJ, Cummins CA, Hunt M et al.,2015). The phylogenic tree with pan-genome matrix was obtained from the phandango software® (https://jameshadfield.github.io/phandango/#/) (Hadfield J, Croucher NJ, Goater RJ et al., 2018). Data were summarized and presented as gene present and absent in a table and hit maps.

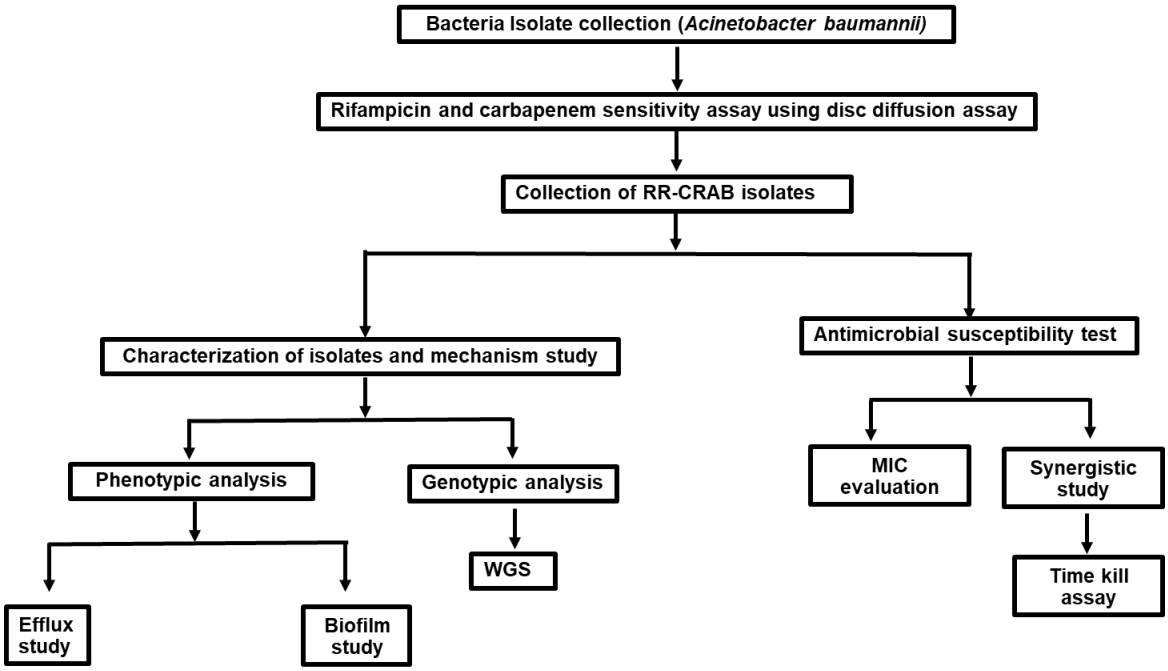


Figure 3. Study workflow / conceptional framework.

CHAPTER 4

RESULTS

4.1 Antibiotic susceptibility test

CRAB clinical isolates (n=218) were obtained from patients with underlying health conditions with a history of prior antibiotics use and admitted for 6 to 32 days. Rifampicin sensitivity test was conducted on 218 CRAB clinical isolates using a disc diffusion assay. Thirty-one representative clinical isolates with rifampicin zone of inhibition between 0-18nm were further analysed by broth micro-dilution assay and the antimicrobial resistant profile of 15 different antibiotics were obtained. The result disclosed that the isolates expressed an increasing antibiotic-resistant rate to almost all the antibiotics used in the study. A total of 16% (5/31) of the isolates were susceptible to rifampicin, 19% (6/31) were intermediate, and 65% (20/31) were resistant. However, approximately 71% (22/31) and 94% (29/31) of the isolates were susceptible to tigecycline and minocycline respectively. The potency of carbapenems, gentamycin, tobramycin, and chloramphenicol were lost among the clinical isolates. In addition, 15% (3/20) of the isolates were susceptible while 85% (17/20) demonstrated resistance against amikacin. The activity of trimethoprim-sulfamethoxazole was also limited among isolates resulting in 19% (5/31) susceptibility and 84% resistance. Precisely, 97% (30/31) and 39% (12/31) of the isolates displayed resistant MICs to ciprofloxacin and levofloxacin respectively while 42% (13/31) were susceptible to levofloxacin. Among the 31 isolates, 20 rifampicin-resistant isolates with MICs between 4 – 256 μg/mL were treated with polymyxin. All the isolates were intermediate to polymyxin B while 75% (15/20) of the

isolates demonstrated intermediate to colistin and 25% (5/20) were resistant. (Figure 4). Notably, the majority of the isolates expressed a high level of resistant to aminoglycoside at MIC ≥ 1024 µg/mL. Due to the high MICs exhibited by the aminoglycosides and the reduced MICs for polymyxin B, amikacin, tobramycin, gentamycin and polymyxin B were dropped from the study.

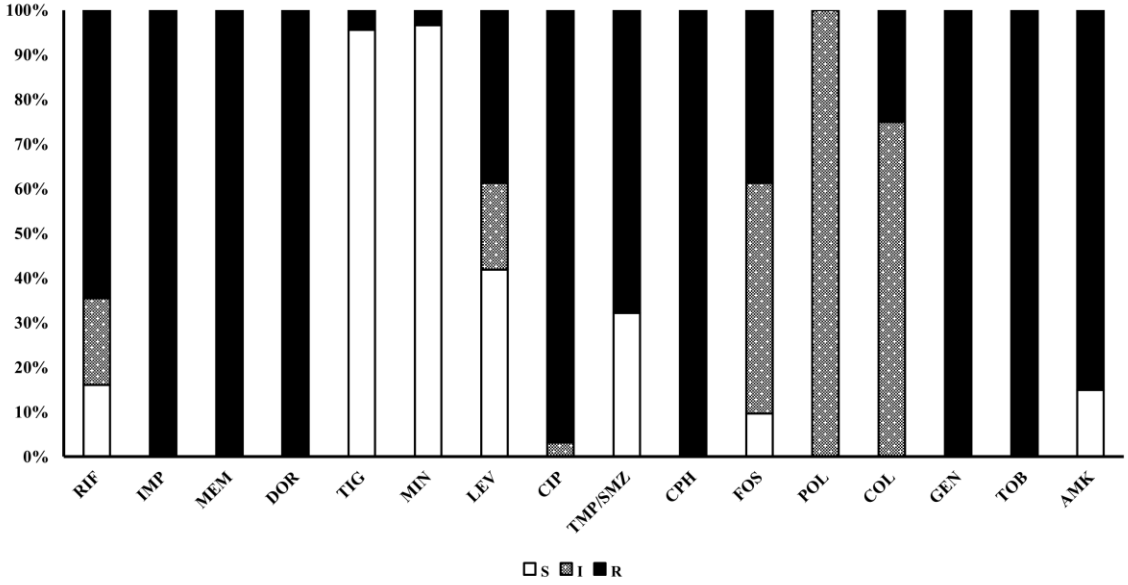


Figure 4. The prevalence rate of antibiotic resistance of *Acinetobacter baumannii*

4.2 Checkerboard assay

A synergism study of rifampicin and other classes of antibiotics was conducted on 31 CRAB clinical isolates and the outcome of the experiment disclosed that 10/31 isolates responded with synergism, 18/31 were addictive, and 3/31 (10%) were indifferent to rifampicin plus imipenem combination. The combination of rifampicin and meropenem resulted in synergism, (23/31) addictive, and (3/31) indifferent in 7/31 (23%), 23/31 (74%), and 3/31 (10%) isolates, respectively. In addition, when rifampicin

was combined with doripenem, the synergistic activity of the combination was noticed in only two isolates. while 27 and 2 out of 31 isolates responded with additive and indifferent respectively (Table 5). Rifampicin combined with tigecycline was synergistic in 4/31 (13%), addictive in 26/31 (84%), and indifferent in 1/31 (3%) isolates. Similarly, the rifampicin and minocycline combination showed synergistic effects against 5/31 (16%), addictive effects against 23/31 (74%), and did not affect 3/31 (10%) isolates (indifferent) (Table 6). When rifampicin was combined with ciprofloxacin, it showed synergistic effects in only 1/31 (3%), addictive effects in 28/31 (90%), and did not affect 2/31 (6%) isolates. Similarly, the combination of rifampicin with levofloxacin demonstrated synergistic effects in 2/31 (6%), addictive effects in 22/31 (71%), and did not affect 7/31 (23%) isolates (Table 7). Rifampicin plus trimethoprim-sulfamethoxazole demonstrated synergism in 1/31 (3%), addictive in 29/31 (94%), and indifferent in 1/31 (3%) isolates. Furthermore, the combination of rifampicin plus fosfomycin resulted in synergism in 3 isolates (10%), addictive in 23 (74%), and indifferent in 5 isolates (16%). Rifampicin was further combined with chloramphenicol and 2 isolates (6%) responded with synergism, three were addictive while 26 (84%) were indifferent to the combination (Table 8). The efficacy of rifampicin plus colistin was then assessed among 9 representative colistin-resistant CRAB clinical isolates, and the combination led to synergism in eight isolates (89%) out of nine (Table 9). The antibacterial susceptibility test of rifampicin and amikacin, gentamicin, or tobramycin revealed a high MIC of the antibiotics hence the antibiotics were dropped from the study (Table 10)

Table 5. The minimal inhibitory concentrations and the fractional inhibitory concentration index of imipenem, meropenem, and doripenem in combination with rifampicin against CRAB clinical isolates.

Isolates	MIC (µg/mL)					FICI		
ID	ZOI	RIF	IMP	MEM	DOR	RIF+ IMP	RIF +MEM	RIF + DOR
ST002	18	2 (I)	32 (R)	32 (R)	16 (R)	1 (Ind)	0.8 (Ad)	0.8 (Ad)
ST004	NZ	64	128 (R)	16 (R)	16 (R)	0.6 (Ad)	0.6 (Ad)	0.6 (Ad)
ST011	7	1 (S)	512(R)	128 (R)	32 (R)	0.8 (Ad)	0.6 (Ad)	0.6 (Ad)
ST016	6	1 (S)	16 (R)	16 (R)	16 (R)	1 (Ind)	0.8 (Ad)	0.8 (Ad)
PA025	18	1 (S)	128 (R)	64 (R)	16 (R)	0.6 (Ad)	1 (Ind)	1 (Ind)
PA037	17	2 (I)	128 (R)	128 (R)	64 (R)	0.5 (Syn)	0.5 (Syn)	0.8 (Ad)
TR009	NZ	64 (R)	64 (R)	128 (R)	16 (R)	0.5 (Syn)	0.8 (Ad)	0.8 (Ad)
TR023	12	2 (I)	128 (R)	128 (R)	32 (R)	0.5 (Syn)	0.5 (Syn)	0.8 (Ad)
TR045	8	2 (I)	128 (R)	128 (R)	64 (R)	0.8 (Ad)	0.8 (Ad)	0.8 (Ad)
TR057	9	4 (R)	128 (R)	64 (R)	32 (R)	0.8 (Ad)	0.5 (Syn)	0.8 (Ad)
TR069	NZ	32 (R)	64 (R)	32 (R)	8 (R)	0.6 (Ad)	0.6 (Ad)	0.6 (Ad)
TR082	NZ	32 (R)	32 (R)	16 (R)	16 (R)	0.6 (Ad)	0.6 (Ad)	0.6 (Ad)
TR119	15	2 (I)	256 (R)	128 (R)	64 (R)	0.8 (Ad)	0.8 (Ad)	0.8 (Ad)
TR121	13	2 (I)	256 (R)	128 (R)	64 (R)	0.5 (Syn)	0.8 (Ad)	0.8 (Ad)
TR123	7	8 (R)	64 (R)	128 (R)	128 (R)	0.5 (Syn)	0.8 (Ad)	0.5 (Syn)
TR125	NZ	64 (R)	16 (R)	16 (R)	8 (R)	0.5 (Syn)	0.5 (Syn)	0.8 (Ad)
TR131	10	4 (R)	32 (R)	32 (R)	8 (R)	0.6 (Ad)	0.5 (Syn)	0.8 (Ad)
SK009	11	256 (R)	64 (R)	128 (R)	64 (R)	1 (Ind)	0.8 (Ad)	1 (Ind)
SK015	NZ	64 (R)	64 (R)	64 (R)	32 (R)	0.6 (Ad)	0.6 (Ad)	0.8 (Ad)
SK024	NZ	16 (R)	64 (R)	128 (R)	16 (R)	0.6 (Ad)	0.5 (Syn)	0.8 (Ad)
SK025	NZ	16 (R)	128 (R)	64 (R)	16 (R)	0.6 (Ad)	0.6 (Ad)	0.8 (Ad)
SK035	7	1 (S)	64 (R)	32 (R)	32 (R)	0.5 (Syn)	0.8 (Ad)	0.5 (Syn)
SK040	NZ	32 (R)	64 (R)	16 (R)	16 (R)	0.5 (Syn)	0.5 (Syn)	0.6 (Ad)
SK052	NZ	64 (R)	128 (R)	32 (R)	32 (R)	0.6 (Ad)	0.6 (Ad)	0.8 (Ad)
SK056	NZ	64 (R)	64 (R)	32 (R)	16 (R)	0.6 (Ad)	0.6 (Ad)	0.8 (Ad)
SK059	NZ	64 (R)	32 (R)	16 (R)	8 (R)	0.4 (Syn)	0.6 (Ad)	0.6 (Ad)
SK065	NZ	32 (R)	128 (R)	32 (R)	16 (R)	0.4 (Syn)	0.6 (Ad)	0.6 (Ad)
SK067	15	64 (R)	256 (R)	64 (R)	32 (R)	0.8 (Ad)	0.6 (Ad)	0.8 (Ad)
SK068	15	1 (S)	128 (R)	128 (R)	32 (R)	0.6 (Ad)	0.6 (Ad)	0.8 (Ad)
PT004	NZ	32 (R)	>256 (R)	128 (R)	32 (R)	0.6 (Ad)	0.6 (Ad)	0.6 (Ad)
PT046	NZ	32 (R)	64 (R)	16 (R)	8 (R)	0.7 (Ad)	0.7 (Ad)	0.6 (Ad)
RBT	≤16	≥4	≥8	≥8	≥8			
%R		100	100	100	100			

*RBT, Resistant Breakpoint; %R, Percentage Resistant; ZOI, zone of inhibition; ND, not determine; NZ, no zone**;** RIF, rifampicin; MEM, meropenem; IMP, imipenem; DOR; doripenem. S, susceptible; I, intermediate; R, resistant; Syn, Synergy; Ad, additive; Ind; indifferent.

Table 6. The minimal inhibitory concentrations and the fractional inhibitory concentration index of tigecycline and minocycline in combination with rifampicin against CRAB clinical isolates.

		MIC (µg/mL)			FICI	
Isolate ID	**ZOI**	**RIF**	**TIG**	**MIN**	**RIF + TIG**	**RIF + MIN**
ST002	18	2 (I)	2 (S)	0.13 (S)	1 (Ind)	1 (Ind)
ST004	NZ	64	0.13(S)	256	0.63 (Ad)	0.75(Ad)
ST011	7	1 (S)	2(S)	2 (S)	0.63 (Ad)	0.75 (Ad)
ST016	6	1 (S)	8 (R)	1 (S)	0.75 (Ad)	0.75 (Ad)
PA025	18	1 (S)	1 (S)	0.13 (S)	0.63 (Ad)	1 (Ind)
PA037	17	2 (I)	4 (I)	2 (S)	0.63 (Ad)	0.63 (Ad)
TR009	NZ	64 (R)	2 (S)	0.13 (S)	0.63 (Ad)	0.63 (Ad)
TR023	12	2 (I)	4 (I)	0.25 (S)	0.75 (Ad)	0.63 (Ad)
TR045	8	2 (I)	2 (S)	4 (S)	0.75 (Ad)	0.75 (Ad)
TR057	9	4 (R)	4 (I)	0.25 (S)	0.63 (Ad)	0.63 (Ad)
TR069	NZ	32 (R)	0.5 (S)	0.25 (S)	0.63 (Ad)	0.75 (Ad)
TR082	NZ	32 (R)	0.5 (S)	0.25 (S)	0.63 (Ad)	0.75 (Ad)
TR119	15	2 (I)	2 (S)	2 (S)	0.75 (Ad)	0.75 (Ad)
TR121	13	2 (I)	4 (I)	4 (S)	0.38 (Syn)	0.5 (Syn)
TR123	7	8 (R)	2 (S)	1 (S)	0.63 (Ad)	0.63 (Ad)
TR125	NZ	64 (R)	0.25 (S)	0.25 (S)	0.63 (Ad)	0.63 (Ad)
TR131	10	4 (R)	4 (I)	8 (I)	0.75 (Ad)	0.38 (Syn)
SK009	11	256 (R)	4 (I)	4 (S)	0.75 (Ad)	0.63 (Ad)
SK015	NZ	64 (R)	1 (S)	1 (S)	0.5(Syn)	0.5 (Syn)
SK024	NZ	16 (R)	0.5 (S)	1 (S)	0.63 (Ad)	1 (Ind)
SK025	NZ	16 (R)	2 (S)	2 (S)	0.38 (Syn)	0.5 (Syn)
SK035	7	1 (S)	4 (I)	0.25 (S)	0.75 (Ad)	0.75(Ad)
SK040	NZ	32 (R)	0.5 (S)	0.25 (S)	0.63 (Ad)	0.5 (Syn)
SK052	NZ	64 (R)	0.5 (S)	1 (S)	0.63 (Ad)	0.63 (Ad)
SK056	NZ	64 (R)	1 (S)	2 (S)	0.63 (Ad)	0.63 (Ad)
SK059	NZ	64 (R)	1 (S)	0.13 (S)	0.63 (Ad)	0.63 (Ad)
Sk065	NZ	32 (R)	2 (S)	1 (S)	0.75 (Ad)	0.63 (Ad)
Sk067	15	64 (R)	2 (S)	2 (S)	0.63 (Ad)	0.63 (Ad)
SK068	15	1 (S)	4 (I)	2 (S)	0.5 (Syn)	0.63 (Ad)
PT004	NZ	32 (R)	0.125 (S)	0.13 (S)	0.75 (Ad)	0.63 (Ad)
PT046	NZ	32 (R)	0.125 (S)	0.13 (S)	0.75 (Ad)	0.75 (Ad)
RBT	≤16	≥4	≥8	≥16		
%R		100	3	3		

*RBT, Resistant Breakpoint; %R, Percentage Resistant; ZOI, zone of inhibition; ND, not determine; NZ, no zone**;** RIF, rifampicin; TIG, tigecycline; MIN, minocycline; S, susceptible; I, intermediate; R, resistant; Syn, Synergy; Ad, additive; Ind; indifferent.

Table 7. The minimal inhibitory concentrations and the fractional inhibitory concentration index of ciprofloxacin and levofloxacin in combination with rifampicin against CRAB clinical isolates.

Isolate ID	ZOI	MIC (µg/mL)			FICI	
		RIF	LEV	CIP	RIF + LEV	RIF + CIP
ST002	18	2 (I)	2 (S)	16 (R)	0.63 (Ad)	0.75 (Ad)
ST004	NZ	64	512 (R)	2 (I)	0.63 (Ad)	0.63 (Ad)
ST011	7	1 (S)	2 (S)	32 (R)	0.63 (Ad)	0.63 (Ad)
ST016	6	1 (S)	2 (S)	16 (R)	0.63 (Ad)	0.75 (Ad)
PA025	18	1 (S)	2 (S)	8 (R)	1 (Ind)	0.63 (Ad)
PA037	17	2 (I)	2 (S)	32 (R)	1 (Ind)	0.63 (Ad)
TR009	NZ	64 (R)	32 (R)	64 (R)	0.63 (Ad)	1 (Ind)
TR023	12	2 (I)	2 (S)	32 (R)	0.75 (Ad)	0.63 (Ad)
TR045	8	2 (I)	4 (I)	32 (R)	0.75 (Ad)	0.63 (Ad)
TR057	9	4 (R)	16 (R)	64 (R)	0.75 (Ad)	0.75 (Ad)
TR069	NZ	32 (R)	2 (S)	16 (R)	0.63 (Ad)	0.63 (Ad)
TR082	NZ	32 (R)	4 (I)	32 (R)	1 (Ind)	0.75 (Ad)
TR119	15	2 (I)	4 (I)	32 (R)	0.63 (Ad)	0.75 (Ad)
TR121	13	2 (I)	4 (I)	32 (R)	0.75 (Ad)	0.75 (Ad)
TR123	7	8 (R)	2 (S)	16 (R)	0.63 (Ad)	0.63 (Ad)
TR125	NZ	64 (R)	4 (I)	16 (R)	0.63 (Ad)	0.75 (Ad)
TR131	10	4 (R)	8 (R)	128 (R)	1 (Ind)	0.5 (Syn)
SK009	11	256 (R)	16 (R)	128 (R)	0.63 (Ad)	0.63 (Ad)
SK015	NZ	64 (R)	16 (R)	64 (R)	0.75 (Ad)	0.63 (Ad)
SK024	NZ	16 (R)	2 (S)	16 (R)	0.75 (Ad)	0.63 (Ad)
SK025	NZ	16 (R)	2 (S)	32 (R)	1 (Ind)	0.75 (Ad)
SK035	7	1 (S)	2 (S)	8 (R)	1 (Ind)	0.75 (Ad)
SK040	NZ	32 (R)	16 (R)	512 (R)	0.5 (Syn)	0.63 (Ad)
SK052	NZ	64 (R)	32 (R)	512 (R)	0.5 (Syn)	0.63 (Ad)
SK056	NZ	64 (R)	64 (R)	512 (R)	0.75 (Ad)	0.63 (Ad)
SK059	NZ	64 (R)	8 (R)	128 (R)	0.63 (Ad)	0.63 (Ad)
SK065	NZ	32 (R)	32 (R)	128 (R)	0.75 (Ad)	1 (Ind)
SK067	15	64 (R)	8 (R)	32 (R)	0.63 (Ad)	0.63 (Ad)
SK068	15	1 (S)	2 (S)	16 (R)	0.75 (Ad)	0.75 (Ad)
PT004	NZ	32 (R)	2 (S)	16 (R)	1 (Ind)	0.63 (Ad)
PT046	NZ	32 (R)	4 (I)	32 (R)	0.75 (Ad)	0.75 (Ad)
RBT	≤16	≥4	≥8	≥4		
%R		100	39	97		

*RBT, Resistant Breakpoint; %R, Percentage Resistant; ZOI, zone of inhibition; ND, not determine; NZ, no zone**;** RIF, rifampicin; LEV, levofloxacin; CIP, ciprofloxacin S, susceptible; I, intermediate; R, resistant; Syn, Synergy; Ad, additive; Ind; indifferent.

Table 8. The minimal inhibitory concentrations and the fractional inhibitory concentration index of chloramphenicol, trimethoprim-sulphamethoxazole, and Fosfomycin in combination with rifampicin against CRAB clinical isolates.

			MIC (µg/mL)			FICI		
Isolates ID	**ZOI**	**RIF**	**TMP/SMZ**	**CPH**	**FOS**	**RIF + TMP/SMZ**	**RIF + CPH**	**RIF + FOS**
ST002	18	2 (I)	0.06/1.19 (S)	64 (R)	128 (I)	0.75 (Ad)	0.5 (Syn)	0.75 (Ad)
ST004	NZ	64	16/304 (R)	32 (R)	128(I)	0.75 (Ad)	0.75 (Ad)	0.63 (Ad)
ST011	7	1 (S)	0.25/4.75 (S)	128 (R)	256 (R)	0.63 (Ad)	0.63(Ad)	0.63 (Ad)
ST016	6	1 (S)	64/1216 (R)	64 (R)	128(I)	0.63 (Ad)	0.63 (Ad)	1 (Ind)
PA025	18	1 (S)	0.03/0.59 (S)	32 (R)	128 (I)	0.75 (Ad)	0.63 (Ad)	1 (Ind)
PA037	17	2 (I)	8/152 (R)	64 (R)	256 (R)	0.63 (Ad)	0.75 (Ad)	0.63 (Ad)
TR009	NZ	64 (R)	8/152 (R)	32 (R)	128 (I)	0.75 (Ad)	0.63 (Ad)	1 (Ind)
TR023	12	2 (I)	8/152 (R)	64 (R)	512 (R)	0.64 (Ad)	0.63 (Ad)	0.63 (Ad)
TR045	8	2 (I)	8/152 (R)	128 (R)	256 (R)	0.75 (Ad)	0.75 (Ad)	0.75 (Ad)
TR057	9	4 (R)	1/19 (S)	64 (R)	128 (I)	0.75 (Ad)	1 (Ind)	0.75 (Ad)
TR069	NZ	32 (R)	32/608 (R)	128 (R)	512 (R)	0.63 (Ad)	0.75 (Ad)	0.75 (Ad)
TR082	NZ	32 (R)	32/608 (R)	128 (R)	256 (R)	1 (Ind)	0.75 (Ad)	0.63 (Ad)
TR119	15	2 (I)	4/76 (S)	64 (R)	256 (R)	0.63 (Ad)	0.75 (Ad)	0.63 (Ad)
TR121	13	2 (I)	8/152 (R)	64 (R)	128 (I)	0.5 (Syn)	0.63 (Ad)	0.75 (Ad)
TR123	7	8 (R)	8/152 (R)	32 (R)	256 (R)	0.63 (Ad)	0.75 (Ad)	1 (Ind)
TR125	NZ	64 (R)	32/608 (R)	64 (R)	128 (I)	0.75 (Ad)	0.63 (Ad)	0.63 (Ad)
TR131	10	4 (R)	2/38 (S)	128 (R)	512 (R)	0.63 (Ad)	0.63 (Ad)	1 (Ind)
SK009	11	256 (R)	16/304 (R)	128 (R)	64 (S)	0.63 (Ad)	0.75 (Ad)	0.75 (Ad)
SK015	NZ	64 (R)	8/152 (R)	64 (R)	64 (S)	0.75 (Ad)	0.63 (Ad)	0.75 (Ad)
SK024	NZ	16 (R)	4/76 (S)	64 (R)	128 (I)	0.63 (Ad)	1 (Ind)	0.75 (Ad)
SK025	NZ	16 (R)	8/152 (R)	64 (R)	256 (R)	0.63(Ad)	0.5 (Syn)	0.75 (Ad)
SK035	7	1 (S)	0.06/1.19 (S)	64 (R)	256 (R)	0.75 (Ad)	0.63 (Ad)	0.5 (Syn)
SK040	NZ	32 (R)	8/152 (R)	16 (R)	128 (I)	0.63 (Ad)	0.63 (Ad)	0.75 (Ad)
SK052	NZ	64 (R)	4/76 (S)	32 (R)	64 (S)	0.63(Ad)	0.63 (Ad)	0.5 (Syn)
SK056	NZ	64 (R)	4/76 (S)	64 (R)	128 (I)	0.63(Ad)	0.63 (Ad)	0.75 (Ad)
SK059	NZ	64 (R)	16/304 (R)	32 (R)	128 (I)	0.63 (Ad)	0.63 (Ad)	0.5 (Syn)
Sk065	NZ	32 (R)	8/152 (R)	64 (R)	128 (I)	0.75(Ad)	0.75 (Ad)	0.63 (Ad)
Sk067	15	64 (R)	8/152 (R)	64 (R)	256 (R)	0.75(Ad)	0.63 (Ad)	0.63 (Ad)
SK068	15	1 (S)	0.25/4.75 (S)	32 (R)	128 (I)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)
PT004	NZ	32 (R)	16/304 (R)	32 (R)	128 (I)	0.75 (Ad)	0.75 (Ad)	0.75 (Ad)
PT046	NZ	32 (R)	2/38 (S)	16 (R)	128 (I)	0.63 (Ad)	1 (Ind)	0.75 (Ad)
RBT	≤16	≥4	≥4/76	≥8	≥64			
%R		100	84	100	39			

*RBT, Resistant Breakpoint; %R, Percentage Resistant; ZOI, zone of inhibition; ND, not determine; NZ, no zone**;** RIF, rifampicin; TMP/SMZ, Trimethroprim-sulphamethaxazole; CPH, chloramphenicol; FOS, fosfomyxin S, susceptible; I, intermediate; R, resistant; Syn, Synergy; Ad, additive; Ind; indifferent.

Table 9. The minimal inhibitory concentrations of polymyxin and colistin and the fractional inhibitory concentration index of colistin in combination with rifampicin against CRAB clinical isolates.

		MIC (µg/mL)			FICI
Isolates ID	**ZOI**	**RIF**	**POL**	**COL**	**RIF + COL**
ST002	18	2 (I)	ND	ND	ND
ST004	NZ	64	1 (I)	1	ND
ST011	7	1 (S)	ND	ND	ND
ST016	6	1 (S)	ND	ND	ND
PA025	18	1 (S)	ND	ND	ND
PA037	17	2 (I)	ND	ND	ND
TR009	NZ	64 (R)	1 (I)	2 (I)	ND
TR023	12	2 (I)	ND	ND	ND
TR045	8	2 (I)	ND	ND	ND
TR057	9	4 (R)	1 (I)	2 (I)	ND
TR069	NZ	32 (R)	2 (I)	4 (R)	0.3 (Syn)
TR082	NZ	32 (R)	2 (I)	4 (R)	0.3 (Syn)
TR119	15	2 (I)	ND	ND	ND
TR121	13	2 (I)	ND	ND	ND
TR123	7	8 (R)	2 (I)	4 (R)	0.3 (Syn)
TR125	NZ	64 (R)	2 (I)	2 (I)	ND
TR131	10	4 (R)	2 (I)	2 (I)	0.5 (Syn)
SK009	11	256 (R)	1 (I)	2 (I)	0.6 (Ad)
SK015	NZ	64 (R)	2 (I)	2 (I)	ND
SK024	NZ	16 (R)	2 (I)	2 (I)	ND
SK025	NZ	16 (R)	2 (I)	2 (I)	ND
SK035	7	1 (S)	ND	ND	ND
SK040	NZ	32 (R)	2 (I)	2 (I)	ND
SK052	NZ	64 (R)	1 (I)	2 (I)	ND
SK056	NZ	64 (R)	2 (I)	2 (I)	ND
SK059	NZ	64 (R)	2 (I)	4 (R)	0.3 (Syn)
SK065	NZ	32 (R)	2 (I)	2 (I)	0.5 (Syn)
SK067	15	64 (R)	2 (I)	2 (I)	ND
SK068	15	1 (S)	ND	ND	ND
PT004	NZ	32 (R)	2 (I)	2 (I)	0.3 (Syn)
PT046	NZ	32 (R)	1 (I)	4 (R)	0.3 (Syn)
RBT	≤16	≥4	≥4	≥4	
%R		100	0	25	

*RBT, Resistant Breakpoint; %R, Percentage Resistant; ZOI, zone of inhibition; ND, not determine; NZ, no zone; POL, polymyxin; COL, colistin S, susceptible; I, intermediate; R, resistant; Syn, Synergy; Ad, additive; Ind; indifferent.

Table 10. The minimal inhibitory concentrations of rifampicin, gentamicin, tobramycin, and amikacin.

		MIC (µg/mL)			
Isolates ID	**ZOI**	**RIF**	**GEN**	**TOB**	**AMK**
ST002	18	2 (I)	ND	ND	0.06
ST004	NZ	64	2048 (R)	2048 (R)	2048 (R)
ST011	7	1 (S)	ND	ND	1024 (R)
ST016	6	1 (S)	ND	ND	512 (R)
PA025	18	1 (S)	ND	ND	≤2 (S)
PA037	17	2 (I)	ND	ND	ND
TR009	NZ	64 (R)	>2048 (R)	>2048 (R)	>2048 (R)
TR023	12	2 (I)	ND	ND	ND
TR045	8	2 (I)	ND	ND	ND
TR057	9	4 (R)	ND	ND	>2048 (R)
TR069	NZ	32 (R)	>2048 (R)	>2048 (R)	ND
TR082	NZ	32 (R)	2048 (R)	>2048 (R)	ND
TR119	15	2 (I)	ND	ND	ND
TR121	13	2 (I)	ND	ND	ND
TR123	7	8 (R)	ND	ND	ND
TR125	NZ	64 (R)	>2048 (R)	>2048 (R)	ND
TR131	10	4 (R)	ND	ND	ND
SK009	11	256 (R)	ND	ND	>2048 (R)
SK015	NZ	64 (R)	>2048 (R)	>2048 (R)	2048 (R)
SK024	NZ	16 (R)	>2048 (R)	>2048 (R)	>2048 (R)
SK025	NZ	16 (R)	>2048 (R)	>2048 (R)	2048 (R)
SK035	7	1 (S)	ND	ND	≤2 (S)
SK040	NZ	32 (R)	>2048 (R)	>2048 (R)	2048 (R)
SK052	NZ	64 (R)	>2048 (R)	>2048 (R)	2048 (R)
SK056	NZ	64 (R)	>2048 (R)	>2048 (R)	2048 (R)
SK059	NZ	64 (R)	>2048 (R)	>2048 (R)	>2048 (R)
SK065	NZ	32 (R)	2048 (R)	>2048 (R)	>2048 (R)
SK067	15	64 (R)	ND	ND	ND
SK068	15	1 (S)	ND	ND	>2048 (R)
PT004	NZ	32 (R)	>2048 (R)	>2048 (R)	>2048 (R)
PT046	NZ	32 (R)	>2048 (R)	>2048 (R)	>2048 (R)
RBT	≤16	≥4	≥16	≥16	≥16
%R		100	100	100	85

*RBT, Resistant Breakpoint; %R, Percentage Resistant; ZOI, zone of inhibition; ND, not determine; NZ, no zone: RIF, rifampicin; AMK, amikacin; GEN, gentamycin; TOB, tobramycin.; FICI, fractional inhibitory concentration index S, susceptible; I, intermediate; R, resistant.

4.3 Time kill kinetics.

The time-kill assay was conducted to investigate the killing dynamics of the antibiotic combinations. A total of 13 representative isolates that have demonstrated different levels of resistance to rifampicin at MICs ≥ 2 were selected. The results revealed both bacteriostatic and bactericidal effects of combinations of carbapenems with rifampicin. For carbapenems, the seven isolates used in the time-kill assay were selected from isolates with high rifampicin MICs (≥16 µg/mL) and lower rifampicin MICs (2–8 µg/mL). The activity of rifampicin in combination with imipenem was assessed against 6 isolates that have demonstrated increased antibiotics resistance. The results revealed that among the isolates, the combined concentrations have no effect on SK015. For TR069, the combinations were bactericidal at MIC of rifampicin plus 1/2 MIC of imipenem, 1/2 MIC of rifampicin and 1/2 MIC of imipenem, and 1/2 MIC of rifampicin and 1/4 MIC of imipenem. Moreover, rifampicin with imipenem was only bactericidal against ST004 at MIC of rifampicin + 1/2 MIC of imipenem, with lower concentrations being ineffective. At MICs of rifampicin plus 1/2 MIC of imipenem, 1/2 MIC of rifampicin and 1/2 MIC of imipenem, and 1/2 MIC of rifampicin and 1/4 MIC of imipenem against TR131 and TR057, the combination had a bactericidal effect and caused a 3-log decrease in CFU/mL. At MICs of rifampicin plus 1/2 MIC of imipenem, 1/2 MIC of rifampicin, and 1/2 MIC of imipenem antibiotic concentrations, the combination was bactericidal against TR123 while other concentrations were least effective (Figure 5A-F).

The combination of rifampicin plus meropenem was less potent against isolates SK015, TR069, and ST004 resulting in bacteriostatic effect at almost all the

different concentrations. However, at MIC rifampicin + 1/2 MIC meropenem and 1/2 MIC rifampicin + 1/2 MIC meropenem, the combination was bactericidal against SK015 and ST004 with a > 3log reduction in CFU/mL (Figure 6A–C). For SK069, a bactericidal activity of combination was demonstrated at 1/2 MIC rifampicin plus 1/4 MIC meropenem. Rifampicin plus meropenem combination was more potent against the isolate with low rifampicin MIC. A bactericidal activity was observed at varying concentrations of antibiotics resulting in killings at MIC rifampicin plus 1/2 MIC meropenem, and 1/2 MIC rifampicin plus 1/2 MIC of meropenem against isolates TR131, TR123, and TR057. Also, for TR057 a bactericidal effect was observed at 1/2 MIC rifampicin and 1/4 MIC meropenem Figure 6D–F. In addition, the combination of rifampicin and meropenem was bactericidal at 1/4 MIC rifampicin plus 1/4 MIC meropenem and other concentrations against SK024 (Figure 6G).

When rifampicin was combined with doripenem against SK015, TR069, and ST004, the combinations were almost ineffective but at MIC rifampicin plus 1/2 MIC doripenem combination was bactericidal against SK015. The activity of rifampicin plus doripenem was further studied among isolates TR131, TR123, and TR057. The result disclosed that at MIC of rifampicin plus 1/2 MIC of doripenem, 1/2 MIC of rifampicin, and 1/2 MIC of doripenem a bactericidal effect was exhibited. In addition, at 1/2 MIC of rifampicin and 1/4 MIC of doripenem, TR131 and TR0123 responded with bacteriostatic and bactericidal effects respectively. The combination was also bactericidal at 1/4 MIC of rifampicin plus 1/4 MIC of doripenem against TR057 and TR123 (Figure 7A–F).

Further investigations were performed using other classes of antibiotics in combination with rifampicin against TR045, SK009, TR131, TR023, PA037, TR123, SK059. Isolates were resistant or intermediate to both antibiotics used in combination. TR082, TR069, PT046, SK059 which have exhibited increased resistance to almost all the antibiotics were treated with the most effective antibiotics colistin. Rifampicin plus minocycline combinations exhibited bactericidal activity against TR045 at MIC rifampicin plus 1/2 MIC of minocycline and at 1/2 MIC of rifampicin plus 1/2 MIC of minocycline. Combinations of the antibiotics at sub-inhibitory concentrations inhibited bacteria growth with a less than 2 log reduction in CFU (Figure 8A). In addition, the treatment resulted in a bactericidal killing at all the combined concentrations of rifampicin and minocycline against TR131 (Figure 8C). However, the combinations were ineffective against isolates SK009 resulting in a steady growth of the isolates even at MIC rifampicin plus 1/2 MIC of minocycline (Figure 8B). When rifampicin was combined with tigecycline, it resulted in a 2-2.5 log reduction in CFU at all the combined concentrations including at 1/4 MIC rifampicin and 1/4 MIC of tigecycline (Figure 8D & E). For Figure 8F, Combinations resulted in an inhibitory effect at all concentrations with a steady growth of the isolate after 12 h of incubation.

The combined concentrations of rifampicin and ciprofloxacin were bactericidal at MIC rifampicin plus 1/2 MIC of ciprofloxacin, 1/2 MIC of rifampicin plus 1/2 MIC of ciprofloxacin and at 1/2 MIC of rifampicin plus 1/4 MIC of ciprofloxacin against TR023 but bacteriostatic at 1/4 MIC of rifampicin plus 1/4 MIC of ciprofloxacin (Figure 9A). The combination also disclosed a bactericidal activity at MIC rifampicin

plus 1/2 MIC of ciprofloxacin only and an inhibitory activity at other concentrations (Figure 9B & C). Similarly, at MIC rifampicin plus 1/2 MIC of chloramphenicol, a bactericidal effect of combination therapy was demonstrated against TR023 (Figure 9D). Furthermore, steady growth was observed after 12 h of incubation when rifampicin was combined with chloramphenicol (Figure 9E).

The combination of rifampicin with trimethoprim-sulfamethoxazole was less effective against the isolates resulting in a ≤2 log reduction in CFU in a dose-dependent manner (Figure 10 A-C). The effect of the combination of rifampicin with fosfomycin was bactericidal at MIC rifampicin plus 1/2 MIC of fosfomycin but bacteriostatic at 1/2 rifampicin plus 1/2 MIC of fosfomycin, 1/2 MIC rifampicin plus 1/4 MIC of fosfomycin and 1/4 MIC rifampicin plus 1/4 MIC of fosfomycin (Figure 10 D & E). The result suggests that the isolates demonstrated specificity to treatments.

In Figure 11A-D, out of five rifampicin and colistin-resistant CRAB clinical isolates four representative isolates were further investigated to confirm the synergistic activity of colistin with rifampicin against rifampicin-resistant and colistin-resistant isolates. The result disclosed a synergistic activity of the rifampicin plus colistin combination against clinical isolates of CRAB at a sub-inhibitory concentration. Three isolates TR082, TR069, and PT046 were killed at 1/4 MIC of rifampicin and 1/4 MIC of colistin displaying a synergistic and bactericidal effect with ≥ 3 log reduction in CFU/mL (Figure 11A-C). Furthermore, the fractional inhibitory concentration index (FICI) of the three isolates confirms the result of the time-kill assay. However, in isolate SK059 the results did not correlate to treatment at 1/4 MIC of rifampicin and 1/4 MIC of colistin

(Figure 11D) showing an inconsistent result compared with the FICI obtained from the checkerboard assay. Similarly, a previous study has reported a discrepancy between synergism and bactericidal activity of certain antibiotics in combination therapy (Terbtothakun P, Nwabor OF, Siriyong T, *et al*., 2021).

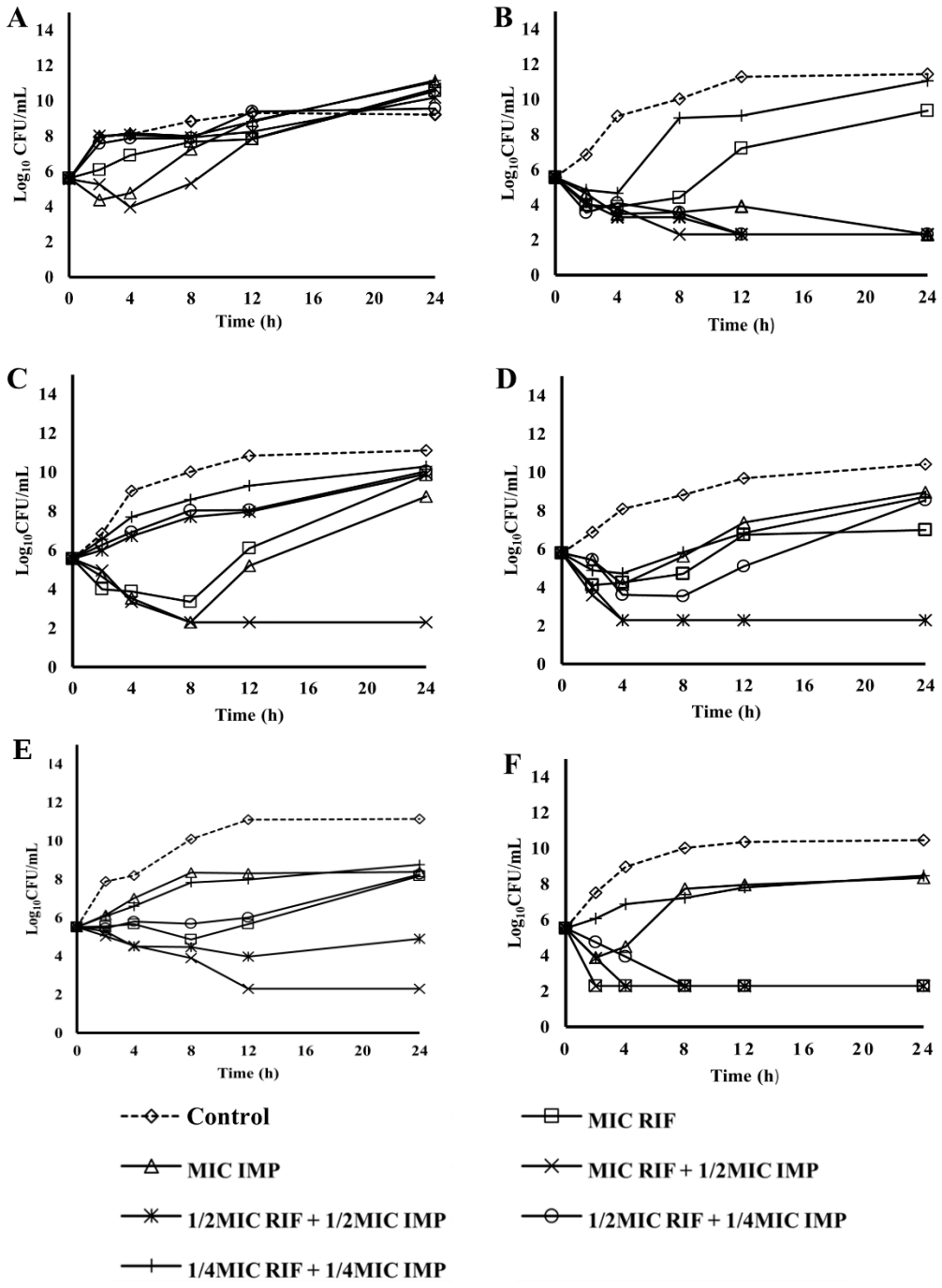


Figure 5. Time-kill curve of rifampicin plus imipenem against rifampicin-resistant and carbapenem-resistant clinical isolates of *A. baumannii*. SK015 (A), TR069 (B), and ST004 (C) are isolates with high rifampicin MIC (≥16 μg/mL), while TR131 (D), TR123 (E), andTR057 (F) are isolates with lower rifampicin MIC (≤8 μg/mL). MIC, minimum inhibitory concentration; RIF, rifampicin; MEM, meropenem.

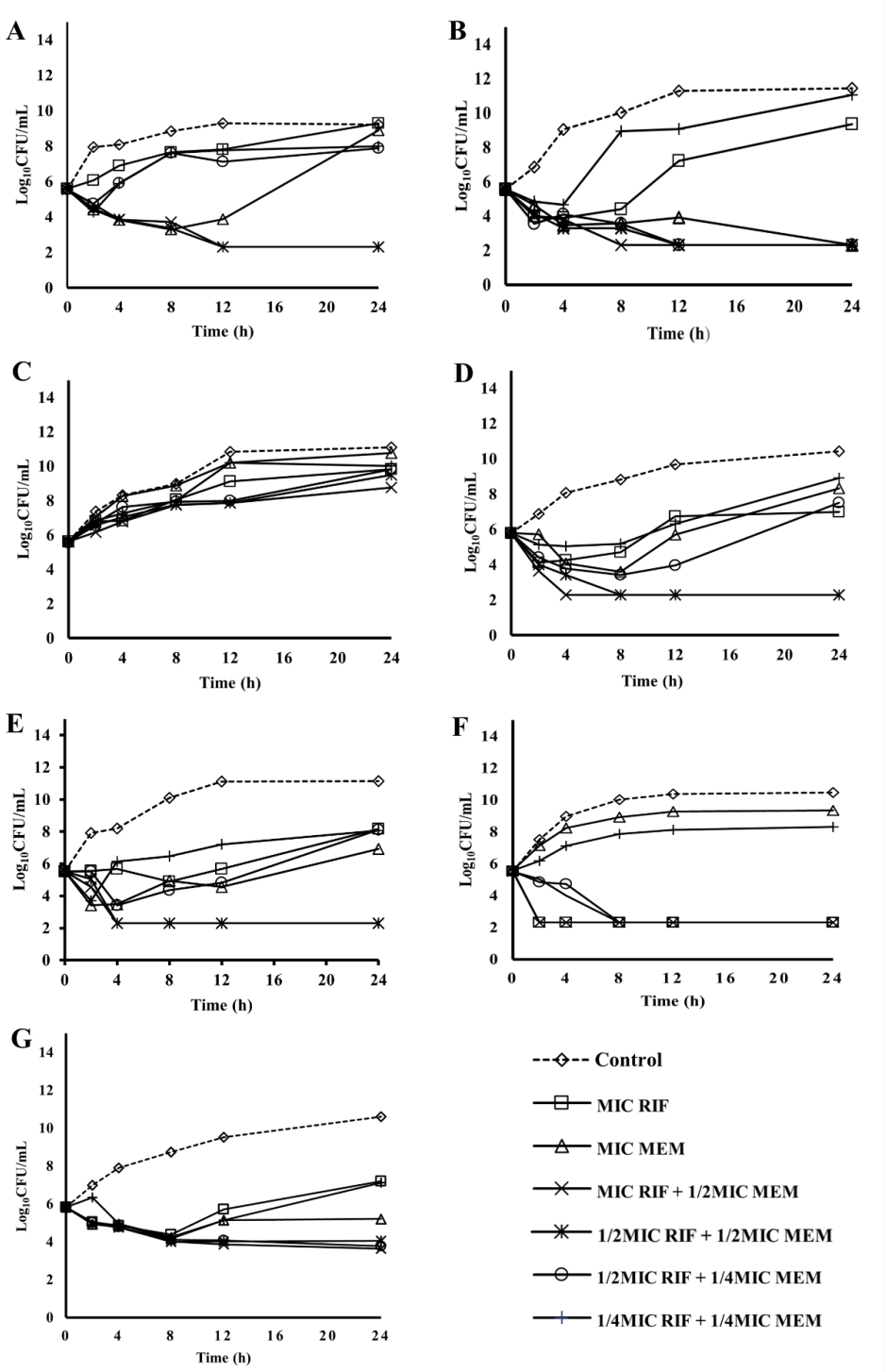


Figure 6. Time-kill curve of rifampicin plus meropenem against rifampicin-resistant and carbapenem-resistant clinical isolates of *A. baumannii*. SK015 (A), TR069 (B), and ST004 (C) are isolates with high rifampicin MIC (≥16 μg/mL), while TR131 (D), TR123 (E), and TR057 (F) and (G) SK024 are isolates with lower rifampicin MIC (≤8 μg/mL). MIC, minimum inhibitory concentration; RIF, rifampicin; MEM, meropenem.

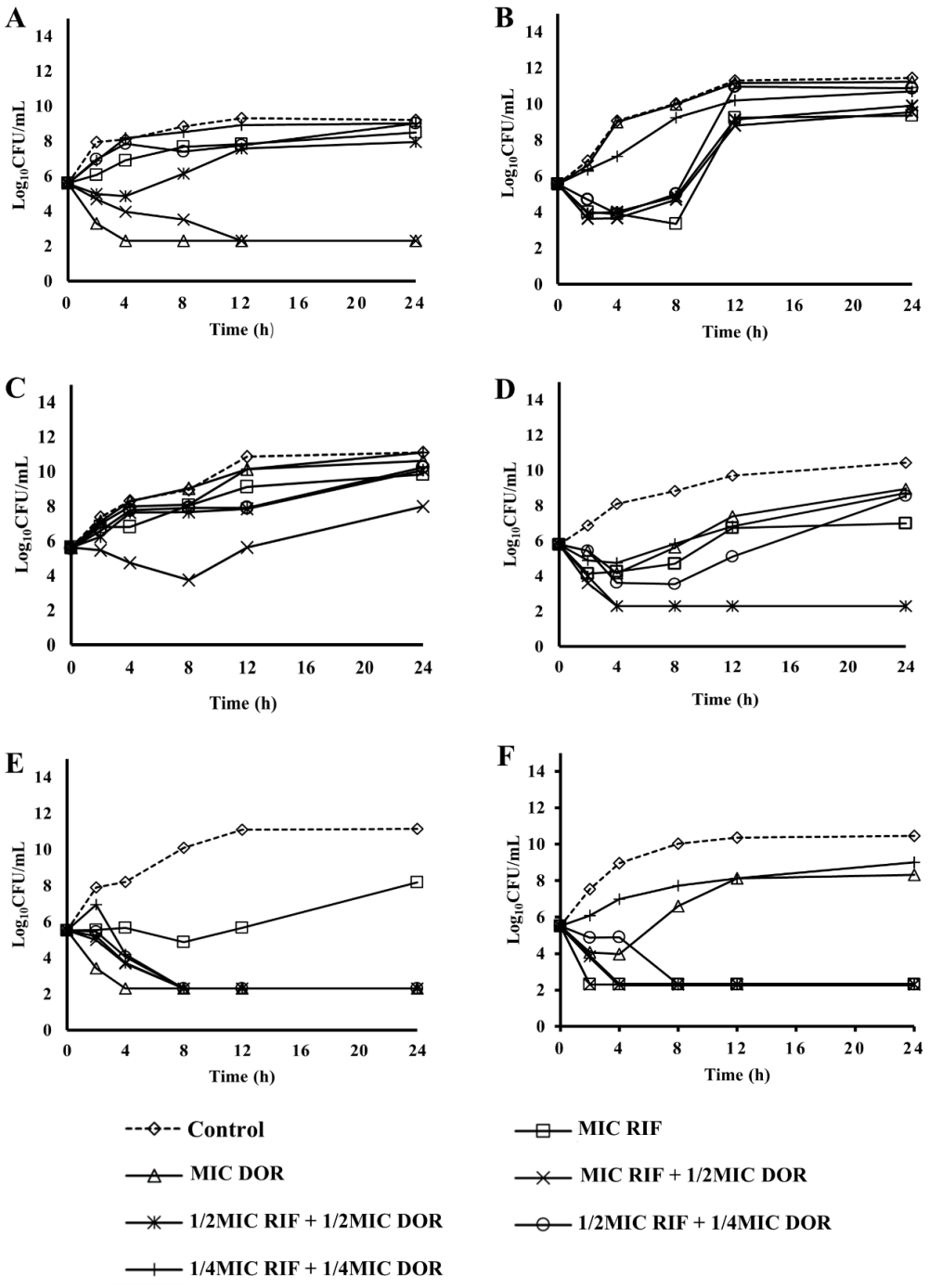
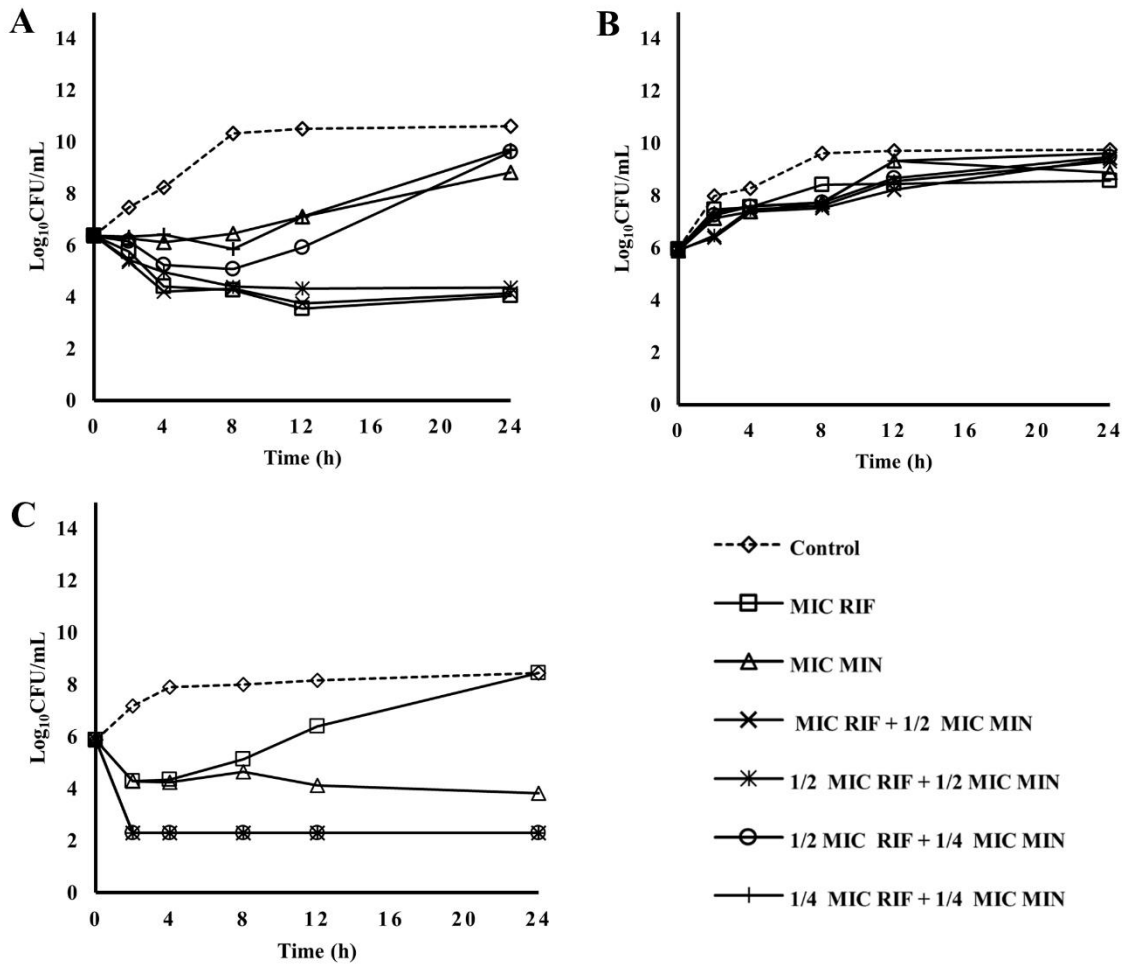


Figure 7. Time-kill curve of rifampicin plus doripenem against rifampicin-resistant and carbapenem-resistant clinical isolates of *A. baumannii*. SK015 (A), TR069 (B), and ST004 (C) are isolates with high rifampicin MIC (≥16 μg/mL), while TR131 (D), TR123 (E), and TR057 (F) are isolates with lower rifampicin MIC (≤8 μg/mL). MIC, minimum inhibitory concentration; RIF, rifampicin; DOR, doripenem.


A
B
C
Log10CFU/mL
Time (h)
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2
4
6
8
10
12
14
0
4
8
12
16
20
24
Control
MIC RIF
MIC MIN
MIC RIF + 1/2 MIC MIN
1/2 MIC RIF + 1/2 MIC MIN
1/2 MIC RIF + 1/4 MIC MIN
1/4 MIC RIF + 1/4 MIC MIN

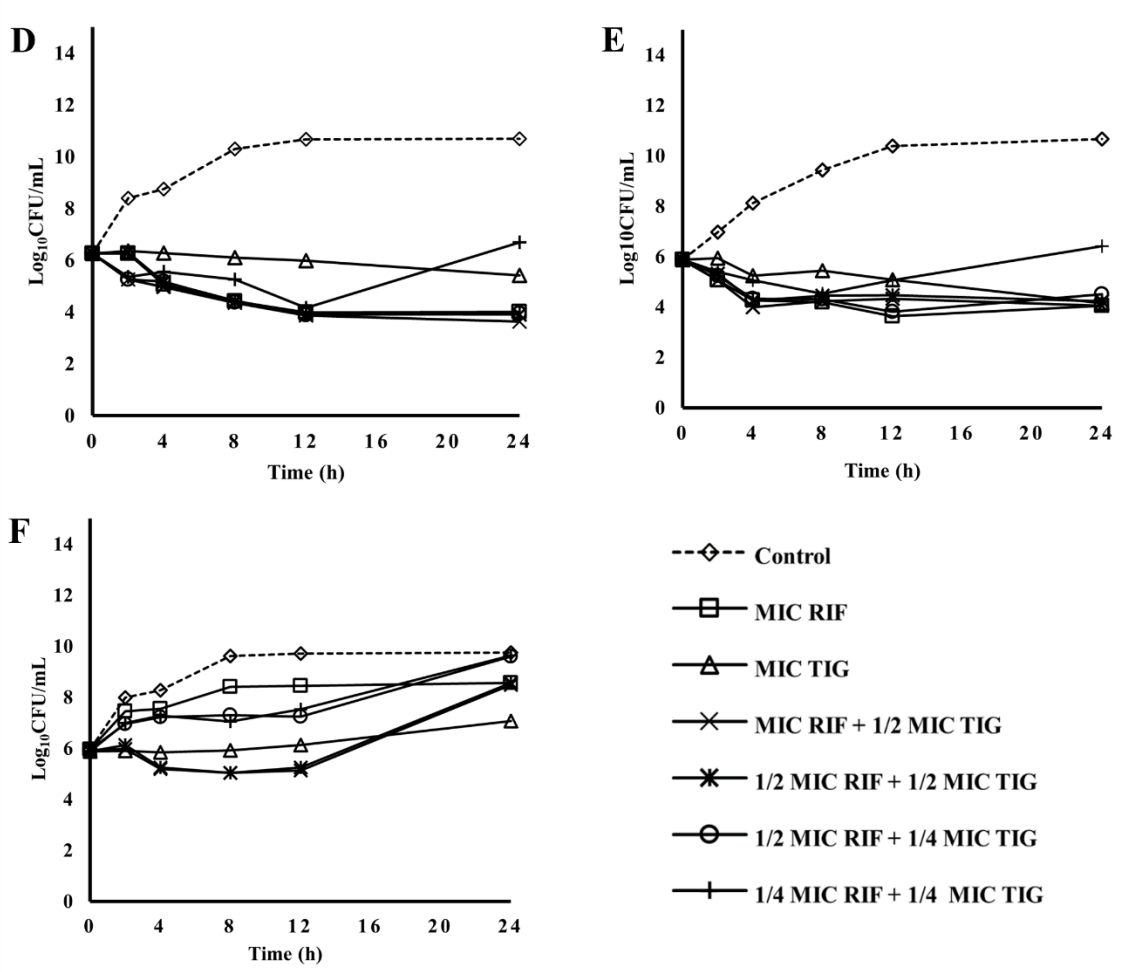
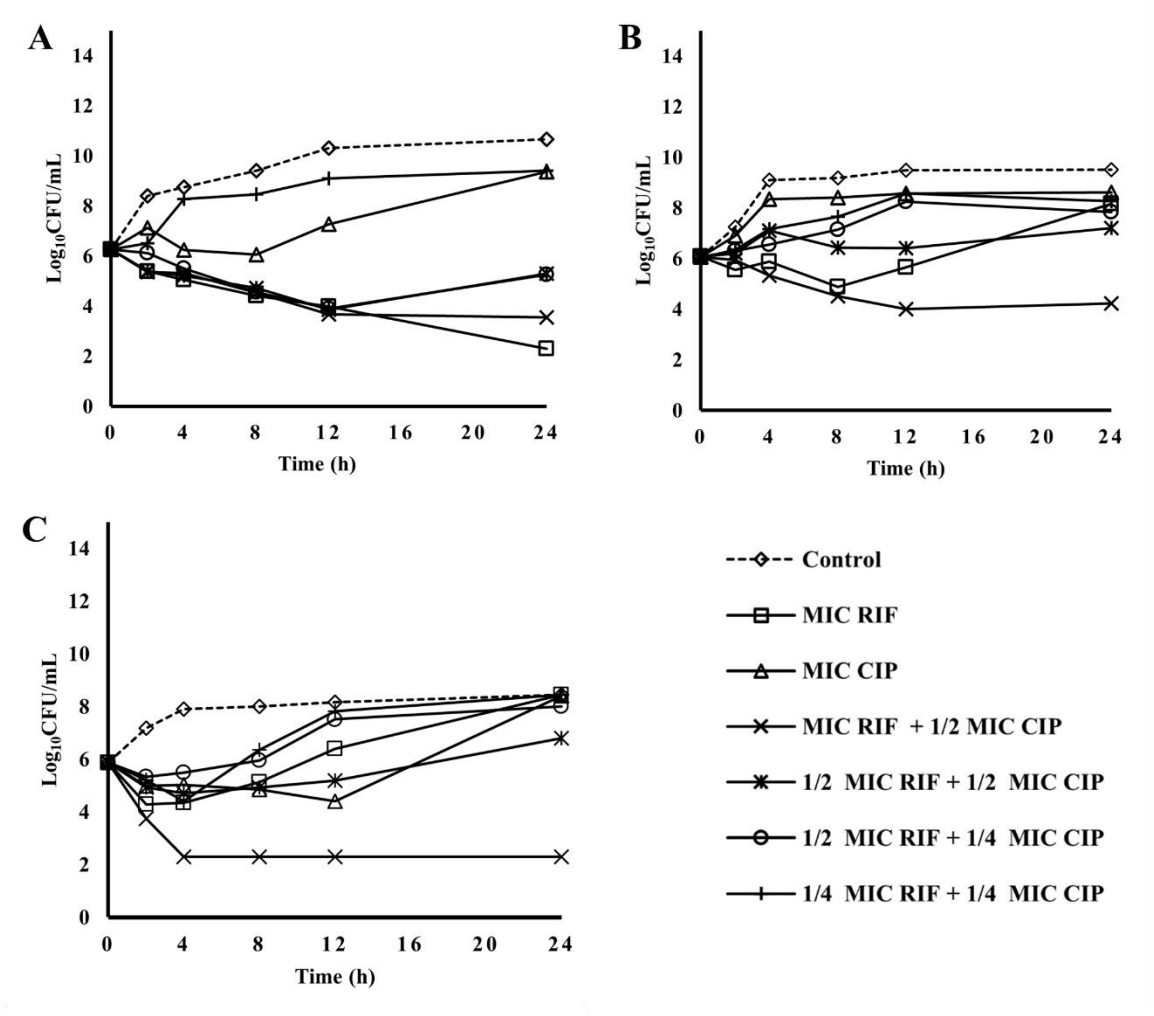


Figure 8. Time killing curve of rifampicin plus tigecycline, or minocycline against CRAB clinical isolates. (A) rifampicin combination with minocycline against TR045 (B) rifampicin combination with minocycline against SK009 (C) rifampicin combination with minocycline against TR131 (D) rifampicin combination with tigecycline against TR023 (E) rifampicin combination with tigecycline against PA037 (F) rifampicin combination with tigecycline against SK009. RIF, rifampicin; TIG, tigecycline; MIN, minocycline.


A
B
C
Log10CFU/mL
Time (h)
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2
4
6
8
10
12
14
0
4
8
12
16
20
24
Control
MIC RIF
MIC CIP
MIC RIF + 1/2 MIC CIP
1/2 MIC RIF + 1/2 MIC CIP
1/2 MIC RIF + 1/4 MIC CIP
1/4 MIC RIF + 1/4 MIC CIP

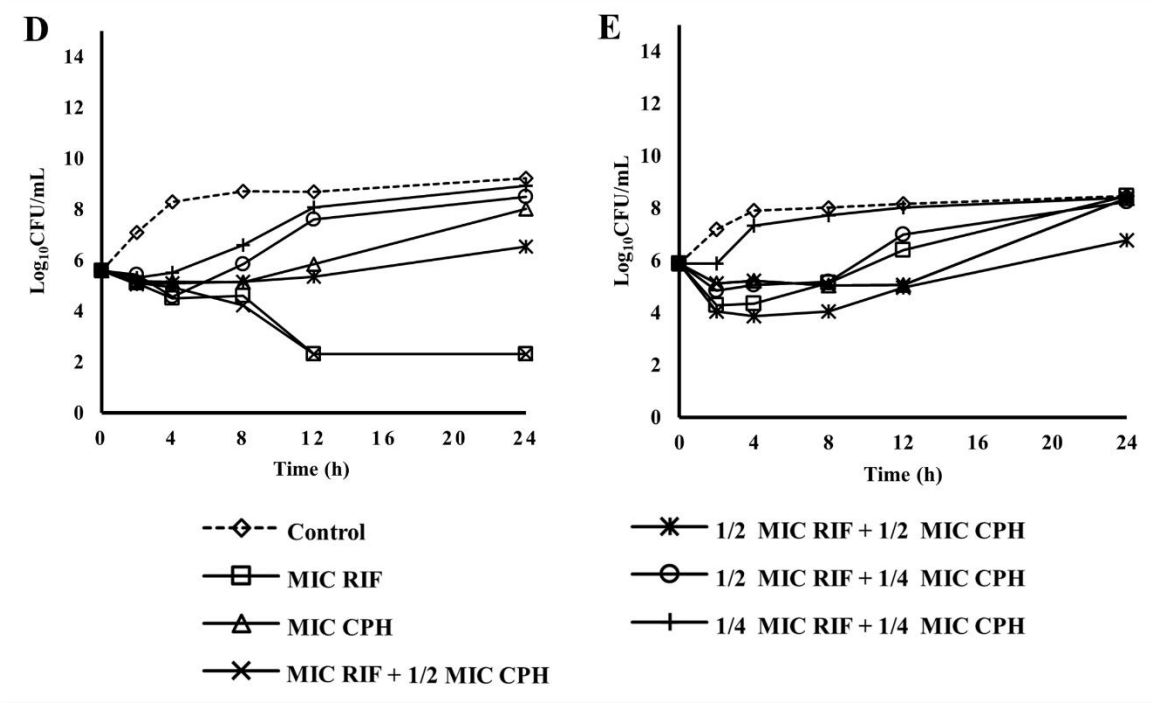
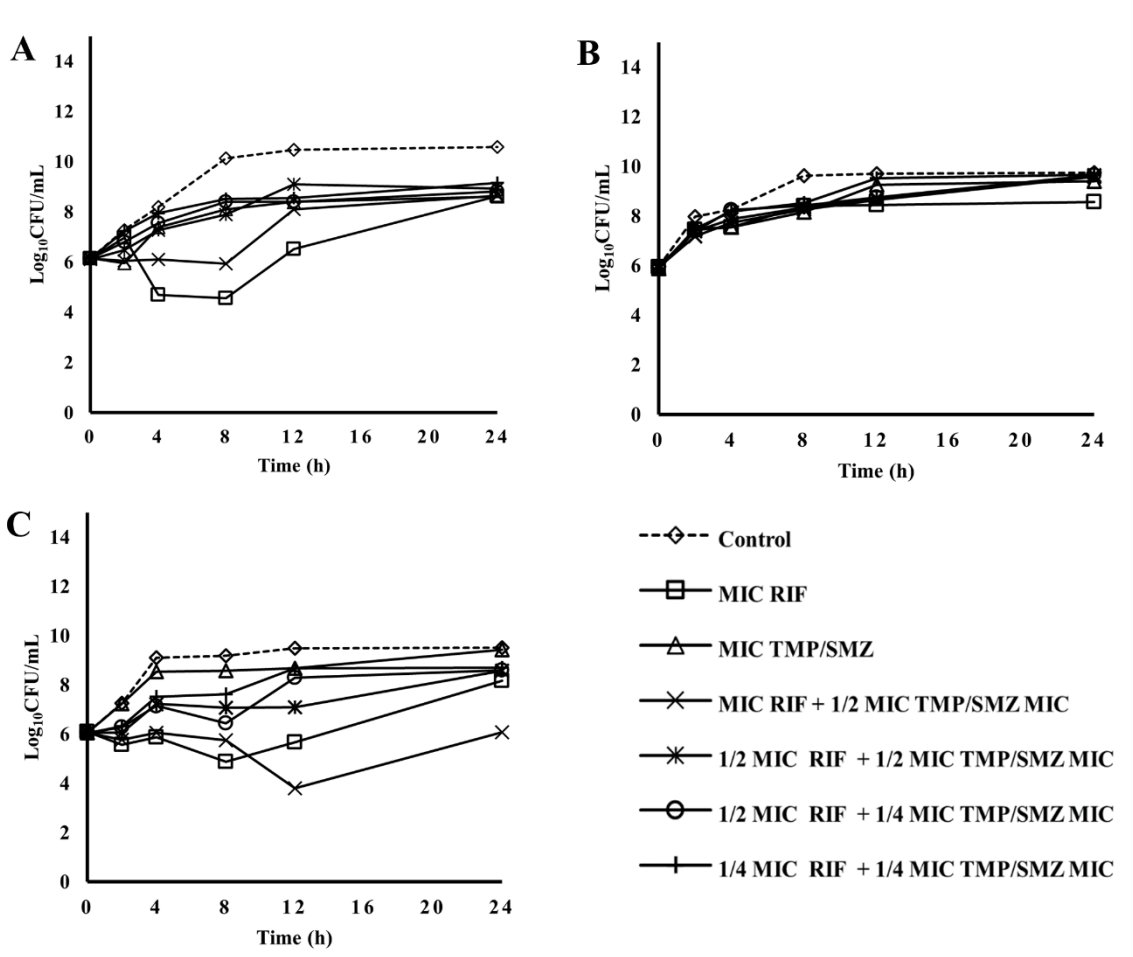


Figure 9. Time killing curve of rifampicin plus ciprofloxacin or chloramphenicol against CRAB clinical isolates. (A) rifampicin combination with ciprofloxacin against TR023, (B) rifampicin combination with ciprofloxacin against TR123, (C) rifampicin combination with ciprofloxacin against TR131 (D) rifampicin combination with Chloramphenicol against TR023 (E) rifampicin combination with Chloramphenicol against TR131. RIF, rifampicin chloramphenicol, CPH; CIP, ciprofloxacin.


A
B
C
Log10CFU/mL
Time (h)
0
2
4
6
8
10
12
14
0
4
8
12
16
20
24
Control
MIC RIF
MIC TMP/SMZ
MIC RIF + 1/2 MIC TMP/SMZ MIC
1/2 MIC RIF + 1/2 MIC TMP/SMZ MIC
1/2 MIC RIF + 1/4 MIC TMP/SMZ MIC
1/4 MIC RIF + 1/4 MIC TMP/SMZ MIC

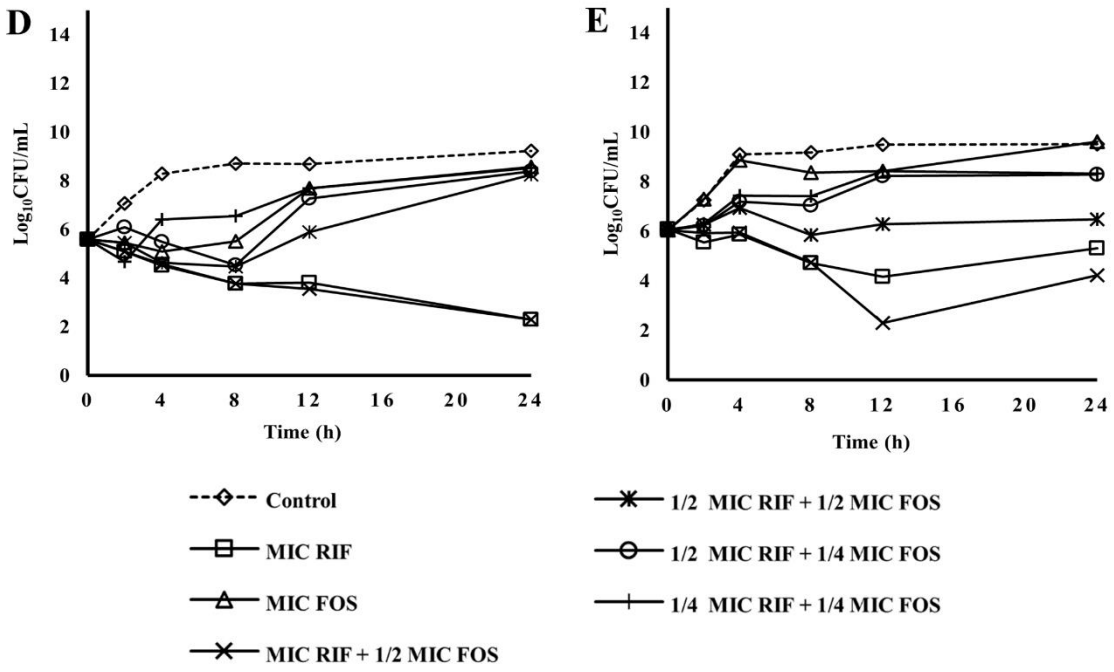


Figure 10. Time killing curve of rifampicin plus trimethoprim-sulfamethoxazole, or fosfomycin against CRAB clinical isolates. (A) rifampicin combination with trimethoprim-sulfamethoxazole against SK059, (B) rifampicin combination with trimethoprim-sulfamethoxazole against SK009, (C) rifampicin combination with trimethoprim-sulfamethoxazole against TR123 (D) rifampicin combination with fosfomycin against TR023 (E) rifampicin combination with fosfomycin against TR123 RIF, rifampicin; TMP/SMZ, trimethoprim-sulfamethoxazole and fosfomycin, FOS.

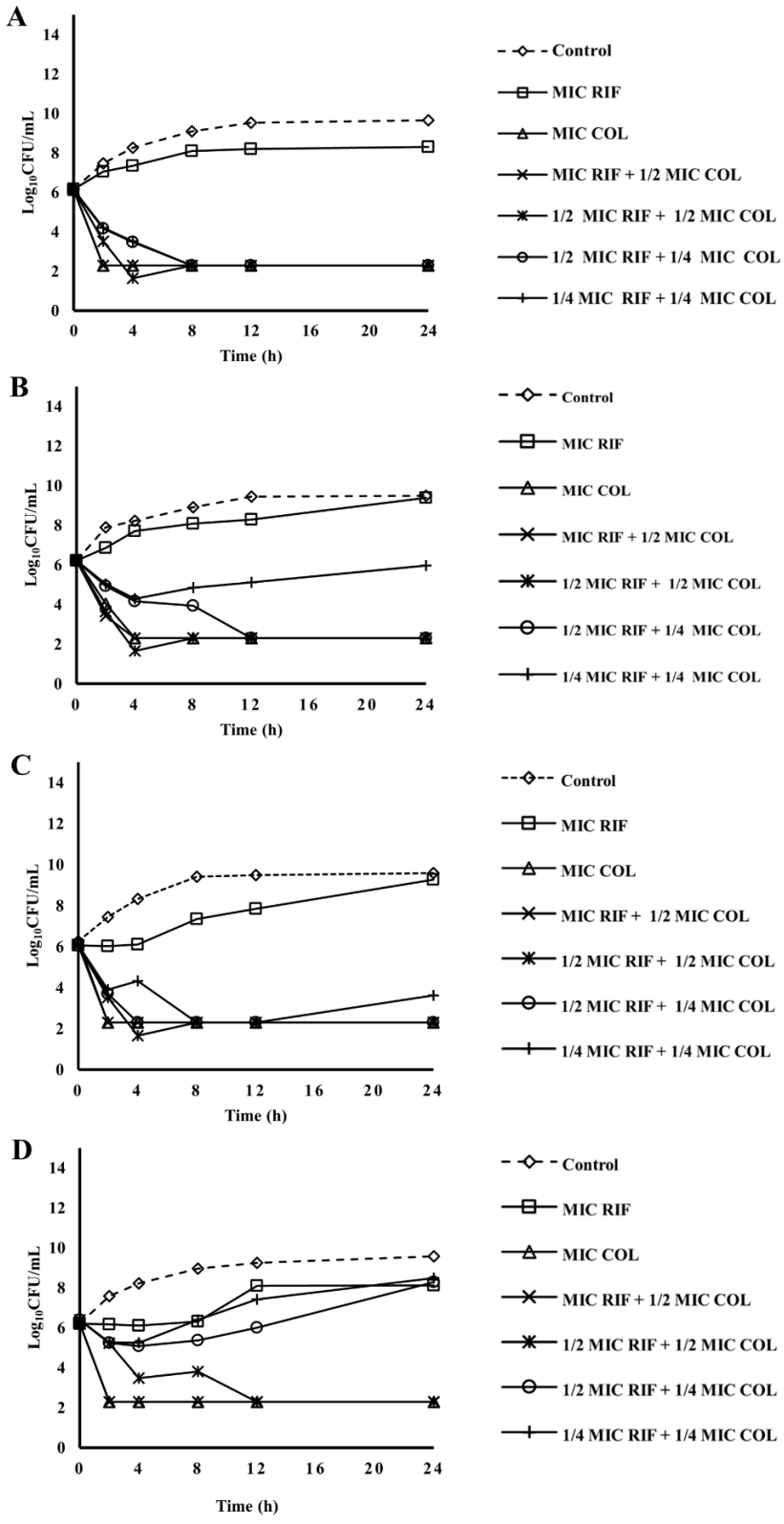


Figure 11. Time killing kinetic of rifampicin combination with colistin against (A) TR082 (B) TR069 (C) PT046, and (D) SK059. RIF, rifampicin; COL, colistin.

4.4 Phenotypic detection of rifampicin-resistant efflux pump

Rifampicin resistance due to the presence of efflux pump phenotype was assessed in the presence of an efflux pump inhibitor carbonyl cyanide chlorophenyl hydrazone (CCCP). Fifteen isolates that demonstrated a high level of rifampicin resistance at MIC ≥ 16 µg/mL were included in the study. SK059 and SK067 were positive to efflux pump phenotype expressing a 4-fold reduction in rifampicin MIC in the presence of CCCP (Table 11).

Table 11. The effect of carbonyl cyanide chlorophenyl hydrazone on rifampicin MIC of *Acinetobacter baumannii* clinical isolates.

Isolates ID	ST	MIC (µg/mL)		Fold reduction
		RIF MIC	RIF+CCCP	
SK009	2	256	128	2
SK015	2	64	32	2
SK024	2	16	16	-
SK056	2	64	16	4
SK059	2	64	32	2
SK065	2	32	32	-
SK067	2	64	16	4
TR009	2	16	16	
TR069	25	32	32	-
TR082	25	32	32	-
TR125	25	64	64	-
PA033	25	32	32	-
ST004	2	64	64	-
PT004	25	32	16	2
PT046	25	32	32	2

ST, sequence type; RIF, rifampicin; CCCP, carbonyl cyanide chlorophenyl hydrazone (efflux pump inhibitor); MIC, minimum inhibitory concentration

4.5 Biofilm eradication effects of rifampicin with carbapenems

Biofilm formation was assessed among 31 CRAB clinical isolates. Based on the crystal violet assay, the result indicated that 100% of the isolates were biofilm formers of which 52% were strong biofilm formers, 34% were moderate biofilm formers and 14% were weak biofilm formers (Table 12). The antibiofilm activities of antibiotics and in combinations were studied on 96 h established biofilm of six strong biofilm-forming isolates including ATCC 19606. The results suggested that at a sub-inhibitory concentration of rifampicin, a 21–68% decrease in biofilm formation was observed compared with untreated biofilm (Figure 12).

Table 12. The characterization and distribution of CRAB clinical isolates based on their ability to form biofilm.

S/N	Isolate	Biofilm forming ability
1	PT046	MBF
2	ST011	MBF
3	PA037	MBF
4	TR023	SBF
5	SK067	SBF
6	ST002	SBF
7	ST010	MBF
8	TR045	MBF
9	PA025	WBF
10	ATCC 19606	SBF
11	TR121	MBF
12	TR057	WBF
13	SK035	WBF
14	PA033	WBF
15	PT004	MBF
16	TR131	SBF
17	TR123	MBF
18	SK068	MBF
19	ST016	MBF
20	SK015	SBF

21	SK024	SBF
22	SK025	SBF
23	SK040	SBF
24	SK052	SBF
25	SK056	SBF
26	SK059	SBF
27	SK065	SBF
28	TR009	SBF
29	TR069	SBF
30	TR082	SBF
31	TR125	SBF
32	ST004	SBF

OD_{cut} =OD_{avg} of negative control + SD of OD of negative control $OD \leq OD_{cut}$ =non-biofilmformers $OD_{cut} < OD \leq 2 \times OD_{cut}$ = weak biofilm formers, $2 \times OD_{cut} < OD \leq 4 \times OD_{cut}$ =Moderate biofilm former, $OD > 4 \times OD_{cut}$ = Strong biofilm former.

The biofilm disrupting activity was then examined on 96 h established biofilm and the viability of biofilm cells was quantified using MTT assay. A concentration-dependent reduction in the number of viable cells was observed for all isolates compared with the untreated control. Treatments with a single antibiotic at 16, 8, and 4 MICs were ineffective against established biofilm compared to antibiotic combinations. Combinations decreased the number of viable cells of 96 h established biofilm at 8 MIC + 4 MIC, 8 MIC + 2 MIC, 4 MIC + 4 MIC, and 4 MIC + 2 MIC rifampicin and carbapenems on all isolates (Figure 13).

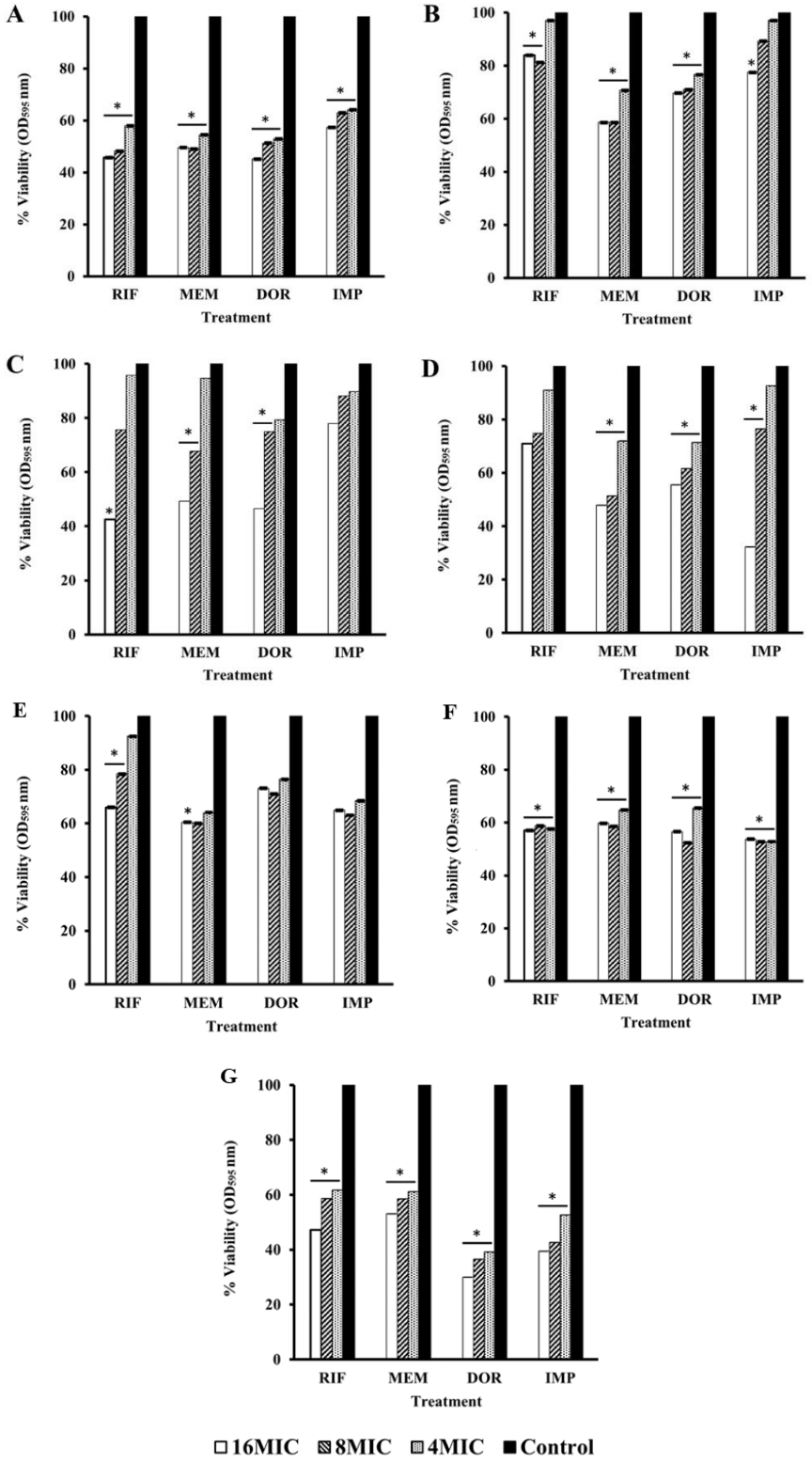


Figure 12. Cell viability of monotherapy against 96 h established biofilm (A) SK015 (B) SK024 (C) TR125 (D) TR045 (E) TR069 (F) ST004 and, (G) ATCC 19606 expressed as percentage viability. MIC, minimum inhibitory concentration; RIF, rifampicin; MEM, meropenem; DOR, doripenem; IMP, imipenem. * $P < 0.05$

The MTT-assay of rifampicin monotherapies on 96 h established biofilm revealed that at 16 MIC, the number of viable biofilm cells reduced by 16–57% among the isolates and ATCC 19606. However, our study indicated that meropenem and doripenem were more effective against *A. baumannii* biofilms (Figure 12). At 16 MIC of meropenem, a 40–52% reduction in viable biofilm cells was observed against the isolates. Doripenem and imipenem single therapy also decreased the viability of biofilm cells in a dose-dependent manner, resulting in 27–70% and 22–68% reduction, respectively. Single antibiotics treatment demonstrated less potency against the tested isolates at higher concentrations, whereas combination at lower concentrations significantly reduced ($P < 0.05$) the viability of biofilm cells (Figure 13).

In the present study, at 4 MIC rifampicin combined with 2 MIC meropenem, the viabilities of biofilms were reduced by 44–74% compared to single antibiotic therapies of either rifampicin or meropenem at 16 MICs (Figure 12). Similarly, 4 MIC rifampicin combined with 2 MIC imipenem, were also more effective than single antibiotic treatments at 16 MIC with a 42–72% reduction in biofilm viability. A combination of rifampicin and doripenem exhibited antibiofilm effects against the 96 h established biofilm, with 44–75% reduction in biofilm viability of the tested isolates and standard strain compared to 16 MIC of doripenem monotherapy (Figure 13).

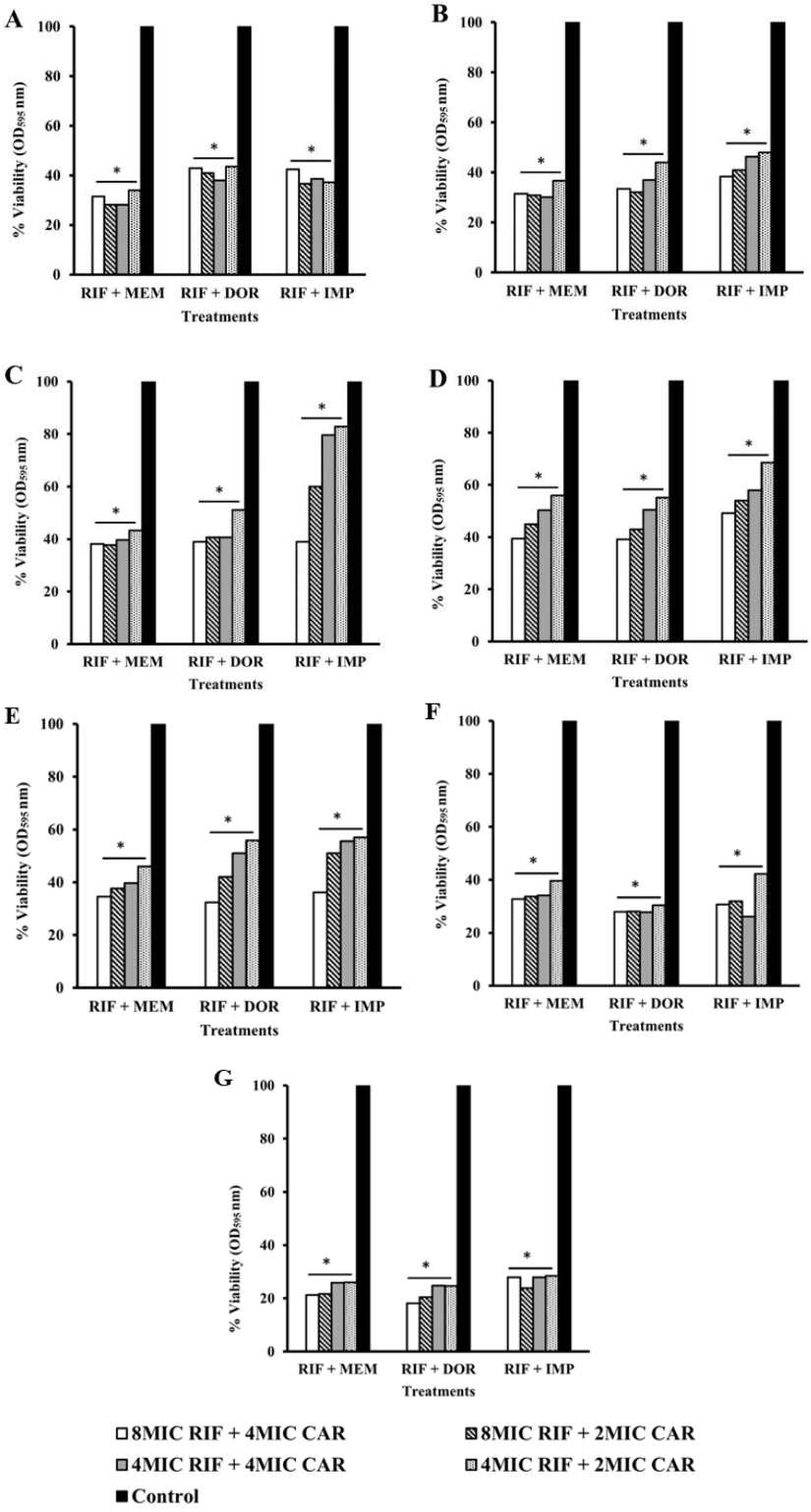


Figure 13. Cell viability of rifampicin combinations with carbapenems against 96 h established biofilm (A) SK015 (B) SK024 (C) TR125 (D) TR045 (E) TR069 (F) ST004 and, (G) ATCC 19606 expressed as percentage viability. MIC, minimum inhibitory concentration; RIF, rifampicin; MEM, meropenem; DOR, doripenem; IMP, imipenem. * $P < 0.05$

4.6 Scanning Electron Microscopy

The activity of antibiotics on the bacteria cell membranes revealed a synergistic disruptive effect of rifampicin and meropenem combination against SK024 (Figure 14). The cell membrane disruption indicates that rifampicin enhances meropenem activity leading to the breakdown of bacterial membranes and an increased influx of antibiotics. Combination therapy at MIC rifampicin and 1/2 MIC meropenem destroyed most of the bacteria cells as shown by the presence of cell debris (Figure 14D). The combination also resulted in changes in the cell structure leading to the elongation of bacteria cells at 1/2 MIC rifampicin and 1/2 MIC meropenem (Figure 14E) after 3 h of incubation. Compared to the control, there was a limited number of bacteria cells when treated with antibiotics in combinations in a concentration-dependent manner. Also, the combination of 1/2 MIC rifampicin and 1/4 MIC meropenem was bactericidal against SK024 after 3 h of incubation.

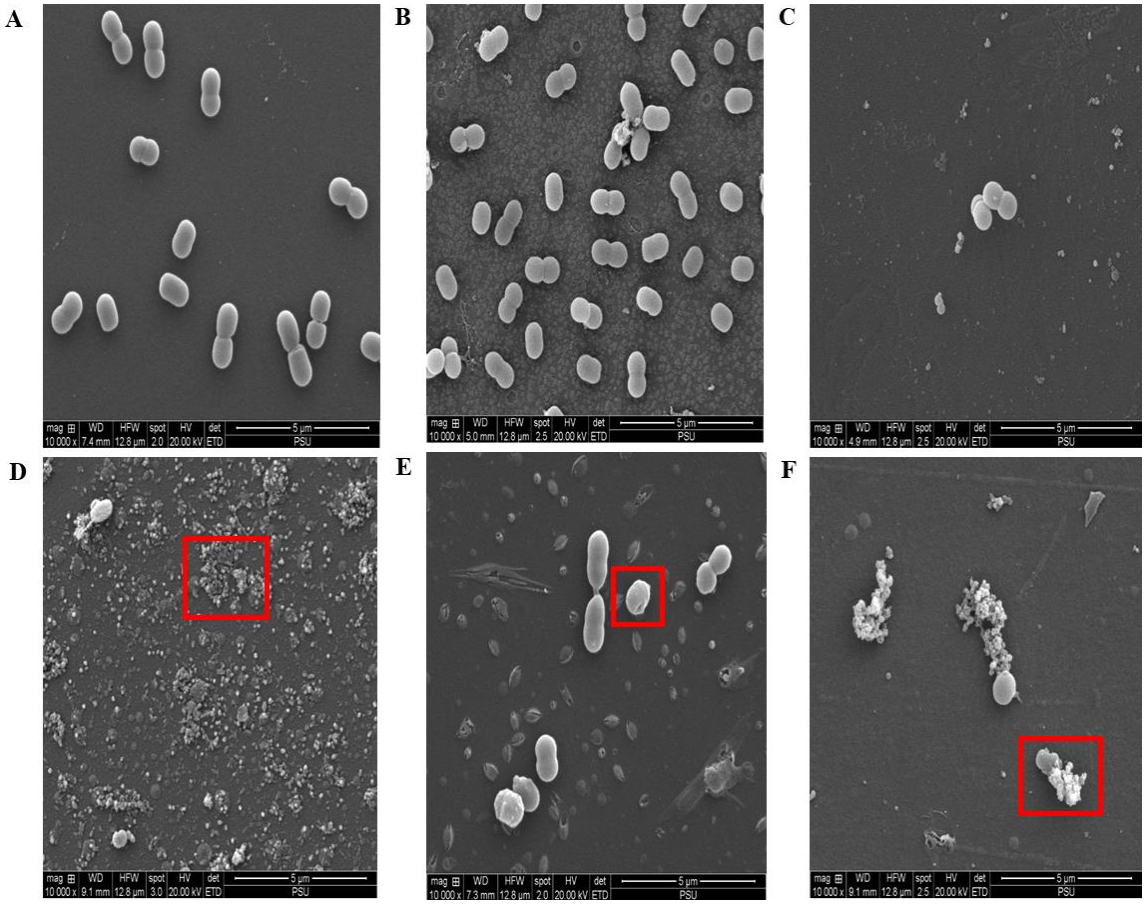


Figure 14. Scanning electron micrograph of the SK024 isolate after 3 h treatment with rifampicin and meropenem alone and in combinations at 10,000 × magnification. (A) untreated cells, (B) treatment with rifampicin alone, (C) treatment with meropenem alone, (D) treatment with MIC rifampicin + 1/2 MIC meropenem, (E) treatment with 1/2 MIC rifampicin + 1/2 MIC meropenem, and (F) treatment with 1/2 MIC rifampicin + 1/4 MIC meropenem.

4.7 Phylogenetic relatedness and sequence types of *A. baumannii* clinical isolates.

The phylogenetic tree aligned with the pan-genome matrix reveals the evolutionary diversity of isolates and their distribution (Figure 15). Comparatively, of the

29 sequences assessed in this study, 66% (19/29), 21% (6/29), 10% (3/29), and 3% (1/29) were ST02, ST025, ST164, and ST016 respectively. Approximately 58% of the pan-genomic matrix was dominated by the accessory genome indicating a progressive evolution of the organisms.

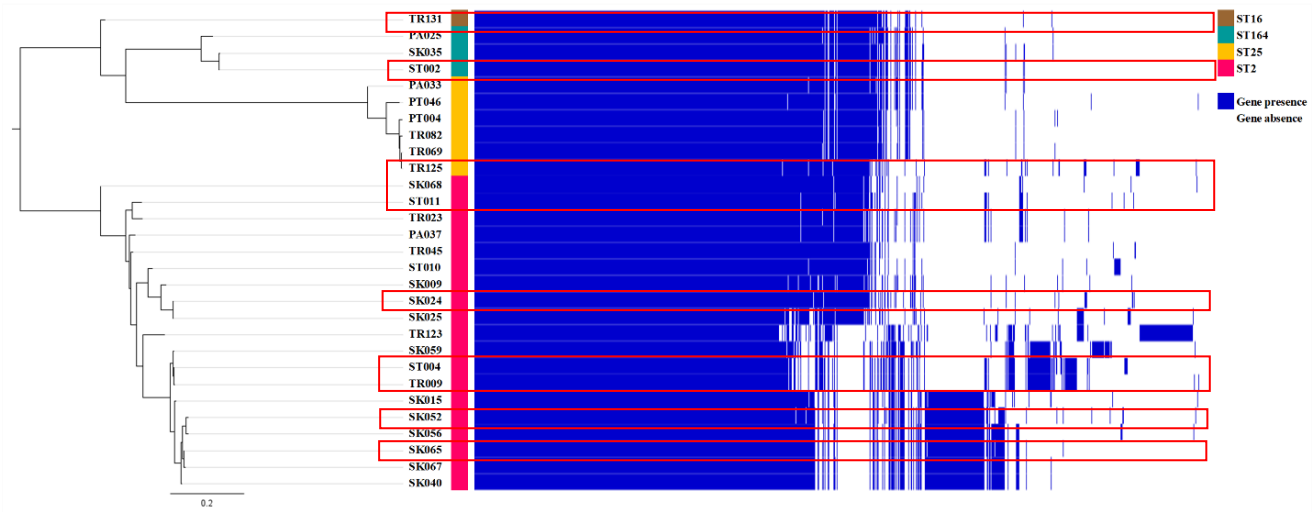


Figure 15. A comparative phylogenetic tree against the pan-genome matrix of 29 *Acinetobacter baumannii* clinical isolates. 19 isolates were assessed from Chukamnerd et al., 2022 and 10 others were sequenced in this study.

4.8 Antimicrobial resistance genes in CRAB clinical isolates

Antibiotic resistance of the isolates may be associated with the presence of different classes of antibiotic-resistant genes. Here, a total of 34 AMR genes were identified among 10 CRAB newly sequenced clinical isolates. The β-lactam inactivating enzymes were predicted, and the blaOXA-23 which might confer resistance to carbapenems was expressed in 9/10 (90%) of the isolates. Other β-lactam resistant related genes including the *bla*$_{\text{ADC-25}}$, *bla*$_{\text{OXA-66}}$, and *bla*$_{\text{PER-7}}$ were also detected with a percentage distribution of 50-70%. About 50% of the isolates harbored the *arr-3* gene while only 1

isolate TR131 was found with the *arr-2* gene resulting in a percentage distribution of exactly (60%) of the rifampicin-resistant mediating genes. Other widely distributed AMR genes identified include the putative aminoglycoside modifying enzyme encoding genes for aminoglycosides acyltransferases (AAC family), aminoglycoside adenyltransferases (ANT family), aminoglycoside phosphotransferases, (APH family) and the nucleotidyltransferase encoding gene aadA1. These enzymes encoding genes were prevalent among the isolates as shown (Figure 4): *aph(6)-Id* (80%), *armA* (80%), *aph(3')-Ia* (50%), *aadA1* (50%) and, *aac(6')-Ib* (40%). Also, *tet(B)* was carried by 60% of the isolates while *tet39* was only present in one out of 10 (10%) of the isolates. The *cmlA1* and *catB8* genes were predicted to be responsible for chloramphenicol resistance and were present in 60% (6/10) and 40% (4/10) of the sequenced genomes respectively. The *aac(6')-Ib-cr*, a bifunctional gene was predicted among 40% (4/10) of the isolates and may be responsible for ciprofloxacin and aminoglycoside-resistant. Although the isolates harbored similar AMR genes, the TR131 was identified with 8 different and unique AMR genes including *aac(3)-IId*, *ant(2'')-Ia*, *aph(3')-VI*, $bla_{\text{OXA-70}}$, $bla_{\text{OXA-58}}$, $bla_{\text{VEB-1}}$, $bla_{\text{NDM-1}}$, and *arr-2*. Additionally, the $bla_{\text{OXA-91}}$ gene was also predicted in ST002 alone (Figure 16).

4.9 Plasmid-mediated antibiotic resistance

The presence of 11 plasmids that may have facilitated the spread of antimicrobial-resistant genes in. *A. baumannii* clinical isolates was disclosed. Five out of the 11 plasmids were highly prevalent including repAci7, repAci3, pS30-1, repM-ci9,

and repAci8. A distribution rate of 50-100% of repAci7, repAci3, pS30-1, repM-ci9, and repAci8 was detected among the 10 isolates. Notably, only SK024, ST002 and TR131 were found harboring p1ABSDF, RepApAB49, and p3ABAYE0002 respectively. Almost all the isolates harbored at least 5 different plasmids while TR125 harbored 3 plasmids. Comparatively, based on data retrieved from the previous genomic analysis and those of the current study repAci7, repAci3, and pS30-1 were highly prevalent among 29 isolates with a percentage distribution of approximately 92% (Table 13).

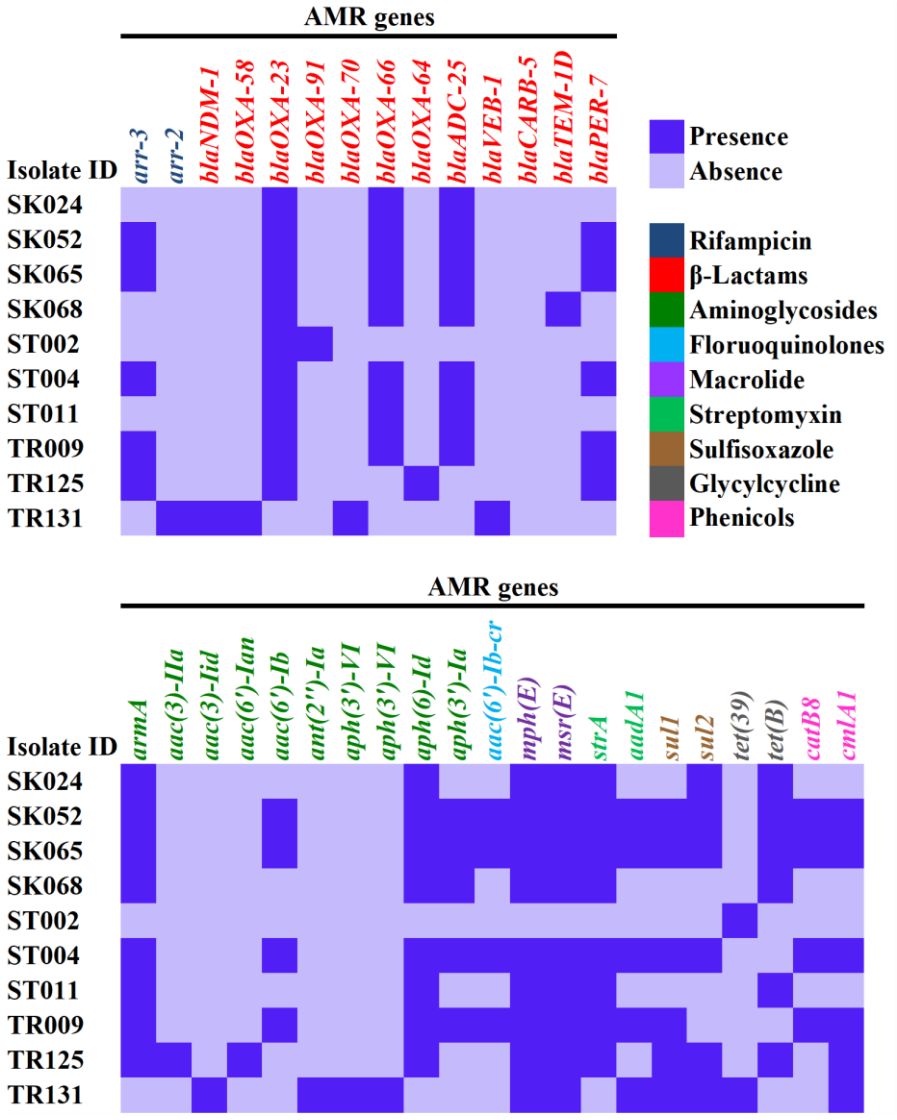


Figure 16. Distribution of antimicrobial resistance (AMR) genes in some ICU patients infected with *A. baumannii* clinical isolates.

Table 13. The distribution of plasmids among 10 *A. baumannii* clinical isolates.

Isolate ID	Sequence Type	Plasmids Predicted
SK024	2	p1ABSDF, repAci2 repAci1, repAci7 pS30-1, repAci3
SK052	2	repAci7, pS30-1 repAci3, repM-Aci9 repAci8
SK065	2	repAci7, pS30-1 repAci3, repM-Aci9 repAci8
TR009	2	repAci7, pS30-1 repAci3, repM-Aci9 repAci8
ST004	2	repAci7, pS30-1 repAci3, repM-Aci90 repAci8
ST002	2	RepApAB49
ST011	2	repAci7, pS30-1, repAci3, repAci1 repAci2
SK068	2	repAci7, pS30-1, repAci3, repAci1, repAci2, pABTJ2
TR125	25	repAci7, pS30-1 repAci3
TR131	16	p3ABAYE0002, pABTJ2, repAci1, repAci8, repM-Aci9

4.10 Virulent factor genes

The ability to acquire virulent genes was then investigated, and 65 putative virulent mediating genes were expressed in the isolates. About 71% of the genes were present in all isolates and 29% were not expressed by all isolates. A repertoire of genes coding for capsular polysaccharides *ACICU_00071–80, ACICU_00086–89,*

ACICU_00091–92, and pgi was disclosed. The genes, *ACICU_00091 –92* and pgi occurred in all the isolates, 80% of the isolates expressed *ACICU_00074 and ACICU_00087*. In addition, *ACICU_00071* and *ACICU_00086* were detected in exactly (7/10) 70% of the isolates whereas 60% of the isolates harbored *ACICU_00071–73, ACICU_00075–77, ACICU_00080* and *ACICU_00088–89.* The genes *ACICU_00078* and *ACICU_00079* were present in only SK024. (Figure 5). In addition, a total of 16 bio-film-associated genes were also revealed. Three AdeFGH RND (resistant nodulation di-vision) efflux pump genes. The abaI and abaR were identified in 100% and 60% of the isolates respectively. Another biofilm-associated gene identified is the bap (biofilm-associated protein) which was present in 8 isolates except for ST002 and TR125. Other biofilm-associated genes including chaperone-usher pathway assembled fimbriae encoded genes (csuA–csuE) and a surface polysaccharide β-(1-6)-poly-N-acetyl-D-glucosamine (PNAG) *pgaA –pgaD* were equally predicted in all the isolates. A well-established iron uptake system composed of 21 siderophore acinetobactin encoding genes including *barA, barB, basA–basD, basF–basJ, bauA–bauF, bfmRS, entE,* and *hemO* were present in almost all the isolates. However, TR125 and TR131 do not express the bauA and hemO siderophore encoding genes respectively. Furthermore, other virulent factor genes responsible for immune evasion and lipopolysaccharide synthesis, (*lpsB, lpxA– lpxD, lpxL, and lpxM*), synthesis of degradative enzymes (*plc*, and *plcD*), cell adhesion and immune evasion (*ompA*) and penicillin-binding protein (*pbpG)* responsible for serum resistance were present in 100% of the isolates (Figure 17).

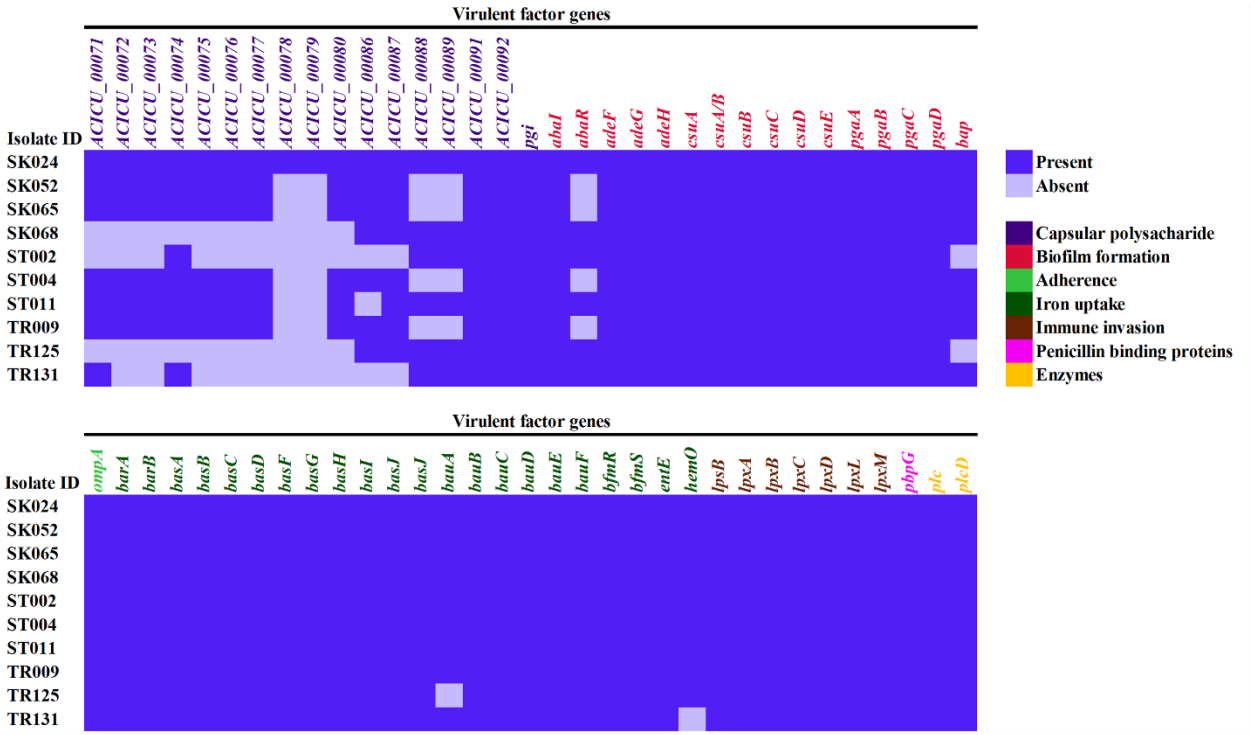


Figure 17. Distribution of virulent genes in some ICU patients infected with A. baumannii clinical isolates.

4.11 Screening for rifampicin resistance due to a chromosomal mutation in the *rpoB* gene

The *rpoB* gene sequence with PubMed ID (NZ_CP015121.1) of *A. baumannii* obtained from the NCBI database was aligned to the genome of the isolates. Single nucleotide polymorphism was detected in 5 out of 29 isolates. The amino acid substitutions were predicted at locations H535Q, S521F, and R788H and may be deleterious against SK024, SK025, SK009, TR131, and TR123 due to the alteration in protein functions (Table 14).

Table 14. Chromosomal mutation in *rpoB* gene with altered protein function.

Isolate ID	Nucleotide	Proteins	% Identity	Molecular mechanisms with P-values ≤0.05
SK024	T1605A	H535Q	99.5	Loss of Allosteric site at R538 Gain of Methylation at K536
SK025	T1605A	H535Q	99.5	Loss of Allosteric site at R538 Gain of Methylation at K536
TR131	G2363A	R788H	99.5	Altered Disordered interface Altered Metal binding Altered Ordered interface Loss of Relative solvent accessibility Altered Disordered interface Altered Transmembrane protein Loss of Ubiquitylation at K785 Loss of ADP-ribosylation at R788 Altered Stability (Loss of Proteolytic cleavage at D790 Loss of Methylation at K785 Loss of Allosteric site at R788
TR123	T1605A	H535Q	99.5	Loss of Allosteric site at R538 Gain of Methylation at K536

4.12 Screening for colistin resistance due to mutations with changes in protein function

The colistin-resistant isolates were further analyzed, and the bacteria genome were aligned to the reference sequence obtained from *A. baumannii* ATCC 19606 with PubMed ID (CP045110.1) and *A. baumannii* ATCC 17978 with PubMed ID (CP053098.1) coding for the PmrCAB two component system, LPS, and EmrAB efflux pump with a percentage identity > 90. Then, mutations associated with colistin resistance with changes in protein functions were predicted. Among the isolates, SK059 was detected with some amino acid substitution in the PmrC and the LpxD at locations K515T and E117K respectively (Table 9). These alterations were predicted to confer

resistance to colistin and have led to changes in the transmembrane protein, gain of ubiquitylation and methylation, and gain of relative solvent accessibility. In addition, the SK059 mutation may be attributed to the lack of bactericidal activity of colistin plus rifampicin at a sub-inhibitory concentration. Notably, three different mutations were predicted for isolate TR123 at location A138T for pmrB, K515T for PmrC, and E117K for lpxD. A slight change in the chromosomal sequence coding for the emrAB efflux pump was linked with colistin resistance in some of the isolates. The modification of emrA_3 and emrB_2 was predicted to cause an alteration in transmembrane protein at F33C and gain of ADP-ribosylation at Q453R in isolates PT046 and TR082 respectively (Table 15).

Table 15. A table showing the distribution of single nucleotide polymorphism of the pmrB, pmrC, pmrD, emrA_3, and emrB_2 genes of *A. baumannii* clinical isolates.

Genes	**Isolates**	**% Identity**	**Nucleotide**	**Protein**	**Molecular mechanisms with P-values ≤0.05**
pmrB	TR123	99.3	G412A	A138T	Altered Transmembrane protein Loss of Relative solvent accessibility Gain of Allosteric site at R134 Gain of Catalytic site at R134
			C1331T	A444V	None
pmrC	TR123 SK059	98.4	A1544C	K515T	Altered Transmembrane protein
lpxD	TR123 SK059	99.7	G349A	E117K	Altered Metal binding Gain of Relative solvent accessibility Gain of Ubiquitylation at E117 Altered Transmembrane protein Gain of Methylation at E117
emrA_3	PA033	95.3	C40T	P14S	None
	PT046		T98G	F33C	Altered Transmembrane protein
	TR069		G103A	V35I	None
	TR082				

emrB_2	PA033	98.1	T386C	M129T	None
	PT046		C412G	P138A	None
	TR069		C625T	L209F	None
	TR082		C627T	L209F	None
			G634A	V212I	None
			G636T	V212I	None
			G721A	V241I	None
			A1358G	Q453R	Gain of ADP-ribosylation at Q453
			A1381G	I461V	None

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

Carbapenem-resistant *Acinetobacter baumannii* (CRAB) burdens global health and standard medical practices with the widespread multidrug-resistant phenotypes. The evolutionary variations of the pathogens by cargoes harbored by mobile genetic elements (MGEs) aggravate the problem through the dissemination of resistance variants that hamper the use of conventional antibiotics (Brito et al., 2022). This study used both phenotypic and genotypic analysis to investigate the resistance mechanisms in CRAB clinical isolates obtained from hospitals in southern Thailand and comparatively assess the spread of resistant determinants within the south poles of Thailand. The study shows that isolates demonstrated multidrug-resistant to various classes of antibiotics including rifampicin. This is in support of a previous study that revealed that rifampicin-resistant clinical isolates are molecular biomarkers for MDR detection (Zewdie, Dabsu, Kifle, & Befikadu, 2020). The 31 isolates utilized in this study demonstrated increasing resistance to almost all classes of antibiotics except for glycylcycline. Similarly, an increased susceptibility rate of CRAB isolates to tigecycline and minocycline has been reported (Brito et al., 2022; Ju et al., 2022). However, the glycylcycline resistance genes are being disseminated and threatening as variants of the *tetB* (tetracycline efflux pump regulatory gene) and *tet39* genes may emerge (Lucaßen et al., 2021). Here, out of the 20 tested isolates, 5 were resistant to colistin at MIC of 4 µg/mL while others were

intermediate. In a present study, an increase in colistin resistance rate of 15% was reported in Thailand (Srisakul et al., 2022). However, in this study, a 25% colistin-resistant rate was exhibited by the isolates. Excluding SK059, for other isolates, the time-kill kinetics result confirms the synergistic study. Similarly, a previous report has demonstrated inconsistency between the time-kill dynamics and the synergism study of certain bacteria isolates (Terbtothakun, Nwabor, Siriyong, Voravuthikunchai, & Chusri, 2021). Recently, some studies have reported a >90% resistance rate of *A. baumannii* to ciprofloxacin, and gentamycin (Hassan & Khider, 2019; Srisakul et al., 2022), and this tallies with the finding of the current study with over 80% resistance rate to the antibiotics. Notably, the limited synergistic activity of rifampicin with other antibiotics may be due to the diverging mechanisms of resistance discovered among isolates. Although, a bactericidal killing of some isolates was achieved when rifampicin was combined with glycylcyclines and carbapenems in the time-kill kinetic most of the antibiotic's combinations were additive to combination therapy. The MICs of the resistant isolates were further investigated in the presence of an efflux pump inhibitor (CCCP) and two isolates SK059 and SK065 expressed a 4-fold reduction in MIC. The finding ascertains that the overexpression of efflux pumps is another mechanism that may confer resistance to rifampicin among *A. baumannii* clinical isolates (Giannouli et al., 2012; Xing, Barnie, Su, & Xu, 2014).

Also, biofilm formation was described as one of the mechanisms conferring resistance to all available antibiotics. Our result demonstrates that all the isolates used in the study were biofilm formers. Similarly, Sarshar *et al*. (2021) reported

that 75 –100% of *A. baumannii* isolates can form biofilms (Sarshar, Behzadi, Scribano, Palamara, & Ambrosi, 2021). This study showed that the antibiofilm activity of rifampicin improved the carbapenem activity against established CRAB biofilm. The combinations of rifampicin with carbapenems inhibited up to 72% viable cells in the 96-h established biofilm at reduced concentration compared with single antibiotics treatment. The results are consistent with a prior study where rifampicin and imipenem were combined (Wang et al., 2014). Generally, combinations of carbapenem with rifampicin resulted in promising antibiofilm effects against *A. baumannii* clinical isolates, suggesting that the combination may be suitable for biofilm eradication. The cumulative effects of the combinations synergistically facilitated the disruption of the bacterial biofilm. Previously, CRAB isolates present in the biofilm were inhibited at high concentrations of single and combined antibiotics compared to that of cells in suspension indicating that biofilm cells are more resistant to antibacterial agents (Shenkutie, Yao, Siu, Wong, & Leung, 2020; Wences et al., 2022). In addition, the limited antibiotic susceptibility in biofilm environments has been linked with the selectivity of antibiotics by bacteria outer membrane structure, reduced antibiotics diffusion due to bacteria aggregation, altered microbial phenotype, and genotypic features during cell-to-cell interaction (Yang, Toyofuku, Sakai, & Nomura, 2017). These cellular changes have been attributed to the increased biofilm eradication concentration of 32-to-256-fold minimum bactericidal concentration of planktonic cells compared to the minimum biofilm inhibition concentration (Shenkutie et al,2020).

The scanning electron microscopy demonstrated and confirmed the synergism observed in the time-kill kinetics of SK024 between rifampicin and meropenem. It further revealed the activity of antibiotics on the bacteria cell membranes and the synergistic disruptive effect of rifampicin and meropenem combination against SK024 (Figure 14). The cell membrane disruption indicates that rifampicin enhances meropenem activity leading to the breakdown of bacterial membranes and an increased influx of antibiotics. This result is in congruence with previous report of rifampicin combination with dalbavancin which is similar to carbapenems by inhibiting bacterial peptidoglycan synthesis (Jacob et al., 2021) Combination therapy at MIC rifampicin and 1/2 MIC meropenem and at sub-inhibitory concentrations destroyed most of the bacteria cell as shown by the presence of cell debris and the structural elongation of the bacteria cells. (Figure 14A-F).

The Genotypic analysis revealed the different resistant patterns of the isolates. Diverse resistant determinant was predominant among isolates despite their sequence types and may be linked to the strain specificity demonstrated by each of the isolates to various antibiotic treatments. The result also revealed that the ST2 is the widest disseminated global clone and indicates a progression in the rise of more variants in the accessory genome replicating a previous study by Chukamnerd *et al*. (2022). The genotypic analysis affirmed the phenotypic and further demonstrates that the isolates were MDR. The presence of 11 different plasmids is an indication that *A. baumannii* clinical isolates can accumulate MGEs that increase the chance of antimicrobial resistance among clinical isolates. This confirms earlier research showing that *A.*

baumannii clinical isolates easily attract MGEs, which results in a high rate of resistance to antibiotics (Partridge, 2011). Based on the high MICs obtained from the antimicrobial susceptibility test, plasmids spread of resistant genes may have led to MDR in the isolates. The variability of the plasmids might also be the cause of the increased resistance to antibiotics of various classes. Moreover, several mobile genetic cargoes were also identified in *A. baumannii* clinical isolates obtained from patients in the intensive care units of hospitals in Thailand (Chopjitt et al., 2020).

Thirty-four different AMR genes were another cause of resistance to almost all the classes of antibiotics used in this study. Similarly, the repAci7, repAci3, and re-pAci1-like plasmids identified in this study were previously described as a carrier of the *bla*$_{\text{OXA-23}}$ gene (Brito et al., 2022). Another recent study evinced that multidrug resistance due to the acquired AMR gene was mainly conferred by different plasmids (Douraghi et al., 2020; Salgado-Camargo et al., 2020). Interestingly, these genes can also mediate resistance to all the choice treatment options recently used against *Acinetobacter spp*. including amikacin, gentamicin, carbapenems, tetracycline, and ciprofloxacin monotherapies. The *bla*$_{\text{OXA-23}}$, a class D carbapenemase was found in almost all the sequenced isolates except for TR131 and in association with *bla*$_{\text{OXA-66}}$, an *Acinetobacter*-derived cephalosporinase *bla*$_{\text{ADC-25}}$ (70%) and *bla*$_{\text{PER-7}}$ (50%). These genes were recently described among the highly prevalent AMR genes in CRAB isolates (Cherubini et al., 2022; Yungyuen et al., 2021). Several aminoglycoside-resistant genes including *aph(6)-Id*, *armA*, *aph (3')-Ia*, and *aadA1* were increasingly spread among isolates. In addition, the presence of different aminoglycoside-resistant genes may be the reason for the high

level of aminoglycoside-resistant among isolates from the antimicrobial susceptibility test conducted. Another study also reported the distribution of these aminoglycoside-modifying enzyme variants (Khurshid et al., 2020; Wongsuk, Boonsilp, Homkaew, Thananon, & Oonanant, 2022). Also, the increasing spread of aminoglycoside-modifying enzyme genes and resistance has been associated with plasmids which promote the bacterial-bacterial spread of aminoglycoside resistance (Bassenden, Rodionov, Shi, & Berghuis, 2016). Our finding revealed that rifampicin resistance among the isolates was majorly due to the spread of the *arr-3* gene which is plasmid-mediated, a chromosomal mutation in the *rpoB* gene, and expression of efflux pump. Notably, there is a limited number of studies addressing rifampicin resistance in *A. baumannii* due to efflux pumps mediated resistance. Further research is needed to investigate the activities of various efflux pumps implicated in *A. baumannii* infection and to characterize efflux pumps associated with rifampicin resistance. Lastly, the transfer of MGEs comprises all mechanisms of resistance including the downregulation or overexpression of some virulent factor genes.

This study also discovered 65 different virulent genes predicted with the ability to increase the pathogenicity of the isolates. Among the virulent genes identified, the absence of abaR regulatory systems in some of the isolates SK052, SK065, TR009, and ST004 may have a detrimental effect by promoting quorum sensing and enhancing biofilm formation. In addition, the formation of biofilm is often associated with elevated pathogenicity of species and increased tolerance to harsh environmental conditions leading to colonization and the manifestation of chronic diseases (Clark, Edgin,

Emerick, & Joshi, 2019; Dexter, Murray, Paulsen, & Peleg, 2015; Gedefie et al., 2021). Also, a study recently discovered that the presence of a biofilm-forming gene in an isolate was associated with the upregulation of the *abaI* gene in the biofilm-forming stage (Li et al., 2021). The implication of excessive expression of virulent factor gene may lead to the dysregulation and function of some cellular features including the AdeFGH efflux system, ACUCI_00071 to 92 which encodes the capsular polysaccharides. The expression level of the ompA gene in all the isolates may also result in carbapenem resistance. Formerly, some research has detected the role of capsular polysaccharide, OmpA, and RND AdeFGH efflux systems modifications in the increased pathogenicity of *A. baumannii* (Douraghi et al., 2020).

Single nucleotide polymorphism with changes in protein function was predicted in some resistant associated genes including the *rpoB* for rifampicin resistance and *pmrB, pmrC, lpxD, emrA_3,* and *emrB_2* for colistin resistance. Mutations in the *rpoB* gene at locations H535Q, R788H, and S521F were detected. Thus, the same changes in the *rpoB* gene have been reported (Giannouli et al., 2012). Several changes were predicted in the isolates and these may probably be associated with the expression of some unique proteins in the matrix (Table 4). Recently, the expression of a different *lpxD, pmrB,* and *pmrC* pattern has been identified in a certain CRAB clinical isolate resistant to colistin (Lunha et al., 2020; Srisakul et al., 2022). In this study, alteration in the protein function was identified at locations A138T of the *pmrB*, (TR123), K515T of pmrC (TR123 and SK059), and E117K of the *lpxD*. The present study identified some changes in the *emrA_3* and *emrB_2* but on the contrary, no EmrAB mutation was

associated with colistin resistance in *A. baumannii* based on a previous report (Gerson et al., 2020), However, this current study evinced that the presence of a mutation in the *emrAB* genes may have contributed to colistin resistance in PT046 and TR082 at location F33C and Q453R respectively.

Multi-targeting antimicrobial technique increases the antibacterial spectrum and narrows the resistance window. The use of rifampicin in combination therapies provides therapeutic benefits by reducing its side effects such as hepatotoxicity, eliminating the emergence of rifampicin resistance, and as well broadening its spectrum of activity. Although the restricted uptake of rifampicin discourages its usage in the treatment of Gram-negative pathogens, combination therapies have demonstrated a reduction in the activity of the outer membrane barrier allowing rifampicin to penetrate the targeted RNA polymerase by synergistically modulating the bioavailability of rifampicin in the presence of other antibiotics such as colistin and carbapenem.

5.2 Conclusion

In this study, our findings indicate that rifampicin plus tigecycline, or minocycline, carbapenem, and colistin demonstrated a synergistic and bactericidal activity against some CRAB clinical isolate with rifampicin MIC $\leq$ 8μg/mL. The antimicrobial effect of rifampicin plus some classes of antibiotics (such as chloramphenicol, ciprofloxacin, trimethoprim-sulphamethoxazole) has proven ineffective for the management of carbapenem-resistant *Acinetobacter baumannii* infection. Rifampicin combination with colistin was the most suitable treatment compared to other

antimicrobials used in this study. Combination therapy with a carbapenem and glycylcyclines also improved the antimicrobial susceptibility of isolates with rifampicin MIC ≤ 8μg/mL. Isolates demonstrated addictive responses to other combinations, and we recommend the use of rifampicin with colistin as the most suitable antibiotic combination against CRAB. The phenotypic analysis of CRAB clinical isolates obtained from southern Thailand showed that the isolates exhibited different responses to antibiotics treatments which may be attributed to the diversity of their genome. Conclusively, the study suggests that rifampicin in combination with these antibiotics may serve as the choice treatment option for some CRAB clinical isolates based on their genetic makeup.

In addition, the antibiofilm activity of rifampicin is of therapeutic advantage and making it a choice treatment option for most biofilm-forming bacteria and MDR organisms as it could curb the spread of the biofilm-producing organisms with a reduced hospital stay in the ICU. Mono-therapeutic antibiotics treatment has been inadequate for the management of biofilm-associated infections due to the diversity associated with biofilm cells, and the inability of most antibiotics to penetrate the strong extracellular polymeric substances scaffold. Administering antibiotics at higher concentrations presents therapeutic risks and may exceed the cytoplasmic threshold leading to the breakdown of vital organs. However, the combination of rifampicin and carbapenem in this study demonstrated improved antibiofilm activity at lowered antibiotic concentrations. Carbapenem and rifampicin showed synergistic activity in inhibiting bacteria biofilm and membrane disruption. The combination reduced the survival rate within the biofilm environment better than the individual antibiotics in a

dose-dependent manner. Although combination therapy did not significantly improve the antimicrobial efficacy when rifampicin was combined with carbapenems, it significantly inhibited biofilm viability suggesting that the concurrent administration of antibiotics in combination may lead to the total eradication of the indwelling population of cells making it easier to kill all unattached cells. CRAB resistance to diverse classes of antibiotics was mostly associated with the emergence of the resistant variant that is presumed to be mediated by various MGEs including plasmids and this may be attributed to the strain specificity of the isolates. A combination of rifampicin with cell wall inhibiting antibiotic provides a suitable antibiotic mechanism against CRAB. Colistin and carbapenem inhibit the cell wall permitting the accumulation of rifampicin which promotes biofilm inhibition and eradication of viable cells in the biofilm. Antibiotic combinations with similar mechanisms of action may serve as a choice treatment option against CRAB.

5.3 Limitations and recommendations

We recommend that the treatment of antibiotics resistance clinical isolates should be specified based on the genomic makeup of the individual isolate and that treatments should be personalized among patients. Secondly, more research should be conducted using other effective cell wall permeabilizer in combination with antibiofilm agents such as rifampicin against CRAB. The study was conducted with a limited number of isolates hence further analysis should be conducted for a more reliable result. Also, it fails to investigate the presence of MGEs such as insertion sequence elements and

transposon. The involvement of the EmrAB efflux system in colistin resistance and rifampicin resistance efflux pump among *A. baumannii* clinical isolates were not well elucidated, therefore, we recommend that more research be conducted to investigate the role of efflux pump in colistin resistance among *A. baumannii* clinical isolates and the characterization of efflux pump responsible for rifampicin-resistant among CRAB.

Statistical Analysis

Dunnett multiple comparison tests were used for the analysis of the data obtained at a significant level of test and control at $P < 0.05$ with the prism software.

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APPENDIX

1.0 Disc diffusion Test of rifampicin against CRAB clinical isolates.

S/N	Isolates	Zones of inhibition (ZOI)
1	SK001	NZ
2	SK015	NZ
3	SK034	NZ
4	SK040	NZ
5	SK049	NZ
6	SK052	NZ
7	SK055	NZ
8	SK056	NZ
9	SK059	NZ
10	SK064	NZ
11	SK065	NZ
12	SK069	NZ
13	ST004	NZ
14	ST026	NZ
15	TR0009	NZ
16	TR0069	NZ
17	TR0082	NZ
18	TR0101	NZ
19	TR0107	NZ
20	TR0125	NZ
21	SK024	5
22	TR0071	5
23	SK025	6
24	ST016	6
25	SK054	6
26	TR0118	6
27	SK035	7
28	ST011	7
29	TR0115	7
30	TR0123	7
31	TR0102	8

32	TR0028	8
33	TR0017	8
34	TR0052	8
35	TR0045	8
36	TR0048	8
37	TR0116	8
38	TR0133	8
39	TR0047	8
40	TR0073	8
41	SK041	8
42	SK053	8
43	SK058	9
44	TR0057	9
45	TR0105	9
46	TR0106	9
47	ST024	9
48	TR0006	9
49	TR0060	10
50	SK044	10
51	SK016	10
52	SK006	10
53	SK050	10
54	ST017	10
55	TR0032	10
56	TR0131	10
57	TR0061	10
58	TR0084	10
59	SK013	10
60	TR0056	10
61	SK002	11
62	SK039	11
63	TR0119	11
64	SK009	11
65	TR0005	11
66	TR0109	11
67	SK077	11
68	TR0044	11
69	TR0074	11
70	TR099	11

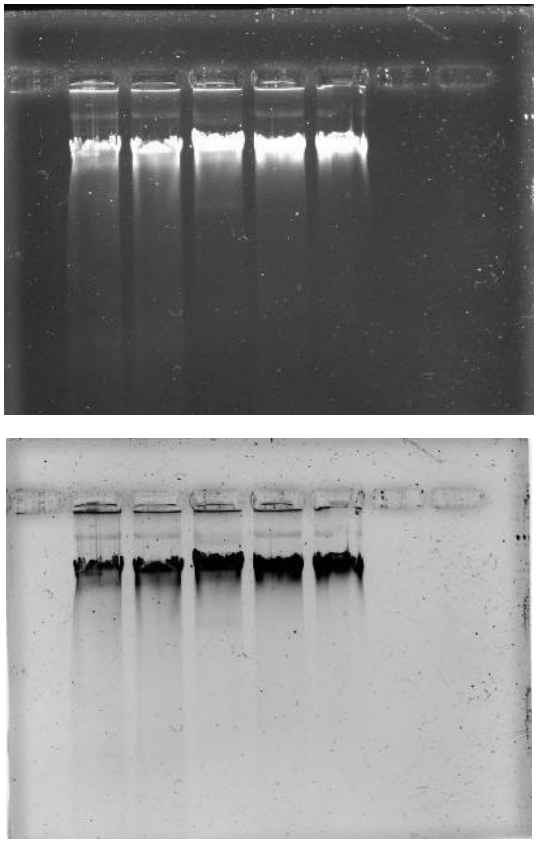
71	ST012	11
72	TR0054	11
73	TR0058	11
74	TR0085	11
75	TR100	11
76	TR0120	11
77	TR0126	11
78	TR0070	11
79	SK022	11
80	TR0046	11
81	TR0035	11
82	SK045	11
83	TR0023	12
84	SK032	12
85	TR0037	12
86	TR0055	12
87	SK010	12
88	SK062	12
89	SK078	12
90	TR0036	12
91	TR0050	12
92	TR0053	12
93	TR0063	12
94	TR0064	12
95	TR0067	12
96	TR0029	12
97	SK028	12
98	TR0062	12
99	TR0068	12
100	SK005	12
101	TR0043	12
102	ST005	12
103	TR0087	12
104	SK057	13
105	ST013	13
106	TR0114	13
107	SK060	13
108	SK061	13
109	TR0059	13

110	TR0089	13
111	SK043	13
112	TR0019	13
113	TR0121	13
114	TR0011	13
115	TR0026	13
116	TR0027	13
117	SK075	13
118	TR0010	13
119	TR0015	13
120	TR0020	13
121	TR0038	13
122	TR0039	13
123	TR0104	13
124	TR0112	13
125	TR0130	13
126	TR0049	13
127	TR0132	13
128	TR0021	13
129	TR0110	13
130	TR0033	14
131	TR0091	14
132	TR0092	14
133	TR0041	14
134	SK042	14
135	SK079	14
136	TR0007	14
137	TR0065	14
138	TR0086	14
139	TR0090	14
140	TR0088	14
141	TR0093	14
142	TR0094	14
143	TR0117	14
144	TR0128	14
145	ST009	15
146	TR0111	15
147	SK067	15
148	SK068	15

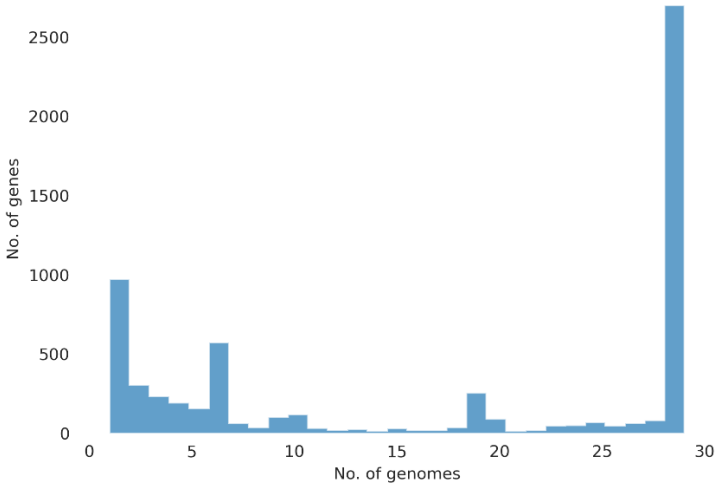
149	TR0124	15
150	ST027	15
151	SK014	15
152	TR0042	15
153	ST021	16
154	TR0113	16
155	TR0022	16
156	SK031	16
157	TR0025	16
158	SK033	17
159	TR0014	17
160	ST002	17
161	TR0122	17
162	SK063	18
163	TR0108	18
164	TR0127	19
165	ST018	20
166	TR0134	25
167	PA033	NZ
168	PA036	11
169	PA037	17
170	PA025	18
171	PA027	18
172	PA032	18
173	PA030	16
174	PT039	15
175	PT034	15
176	PT003	17
177	PT005	19
178	PT006	16
179	PT007	15
180	PT008	16
181	PT009	14
182	PT010	18
183	PT012	15
184	PT018	12
185	PT020	17
186	PT022	12
187	PT022	12

188	PT023	14
189	PT027	16
190	PT028	17
191	PT030	16
192	PT031	15
193	PT034	15
194	PT035	16
195	PT036	15
196	PT037	13
197	PT038	13
198	PT039	15
199	PT040	14
200	PT041	16
201	PT043	15
202	PT044	14
203	PT047	15
204	PT049	18
205	PT050	21
206	PT052	15
207	PT054	15
208	PT058	15
209	PT060	13
210	PT061	15
211	PT062	15
212	PT063	19
213	PT064	17
214	PT071	16
215	PT073	17
216	PT074	15
217	PT078	13
218	PT080	15

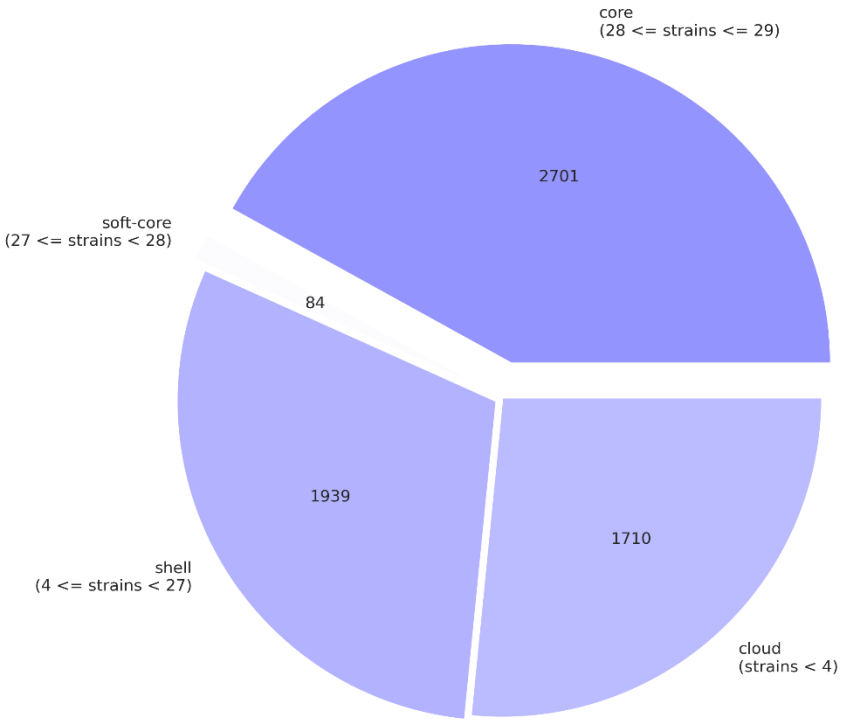
2.0 Agar Rose gel electrophoresis result of bacteria DNA



3.0 Pan- genomic Frequency of 29 CRAB Clinical Isolates



4.0 Pan- genomic Distribution of isolates in the core and accessory Genome



5.0 Demographic and clinical information and outcome of patients

Isolate ID	Hospital	Sample source	Age of patient	Admitted ward	Duration of hospital stay	Underlying diseases						Prior antibiotic use								
						DM	HTN	CKD	CVA	CAD	Pulmonary disease	CRO	CAZ	IMP	MEM	ERT	PIP/TAZ	AMG	LVX/CIP	Other
PA025	Pattani Hospital	Sputum	19	MICU	12	0	1	0	1	1	1	1	1	0	1	1	1	0	1	0
PA037	Pattani Hospital	Sputum	18	SICU	12	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0
PT004	Phatthalung Hospital	Sputum	44	ICU	20	1	0	0	0	0	0	1	1	0	0	0	1	0	0	0
PT046	Phatthalung Hospital	Sputum	35	ICU	21	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1
SK015	Songkhla Hospital	Sputum	55	ICU	25	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0
SK025	Songkhla Hospital	Urine	54	ICU	24	1	0	0	0	0	0	1	1	1	1	0	0	0	0	1
SK035	Songkhla Hospital	Sputum	44	ICU	9	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
SK040	Songkhla Hospital	Sputum	44	ICU	29	0	0	1	0	0	0	1	0	1	0	0	1	0	1	0
SK056	Songkhla Hospital	Sputum	42	ICU	22	1	0	0	0	1	1	1	0	0	1	0	0	0	0	0
SK059	Songkhla Hospital	Sputum	66	ICU	7	1	0	0	0	0	0	1	0	0	1	0	1	0	0	0
SK067	Songkhla Hospital	Blood	24	ICU	8	1	0	0	1	0	0	1	0	0	1	0	1	1	0	0
ST010	Satun Hospital	Sputum	46	ICU	9	1	0	0	1	0	1	0	0	1	1	0	0	0	1	0
TR023	Trang Hospital	Pus	67	ICU	11	1	0	0	1	0	0	0	0	1	0	0	1	0	1	0
TR045	Trang Hospital	Pus	18	ICU	11	1	0	0	0	0	0	0	0	1	0	0	1	0	1	0
TR069	Trang Hospital	Sputum	96	ICU	9	1	0	0	0	0	1	1	0	1	1	0	1	0	0	0
TR123	Trang Hospital	Sputum	22	ICU	13	0	1	0	0	1	0	1	0	1	1	1	1	0	0	1
ST002	Satun Hospital	Sputum	39	ICU	28	0	1	0	0	1	0	0	0	1	1	0	0	0	1	0
ST004	Satun Hospital	Sputum	48	ICU	18	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1
ST011	Satun Hospital	Sputum	56	ICU	19	1	0	1	0	1	0	1	0	0	1	0	1	1	1	0
ST016	Satun Hospital	Sputum	72	ICU	21	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0
TR009	Trang Hospital	Blood	45	ICU	32	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1
TR057	Trang Hospital	Pus	81	ICU	19	1	1	1	0	1	0	1	1	1	0	0	1	0	0	0
TR121	Trang Hospital	Sputum	29	ICU	10	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0
TR125	Trang Hospital	Sputum	38	ICU	7	1	0	0	1	0	0	0	1	1	1	0	0	0	0	0
TR131	Trang Hospital	Pus	42	ICU	6	0	1	1	0	1	0	1	0	1	1	0	1	0	0	1
TR082	Trang Hospital	Urine	56	ICU	12	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
SK024	Songkhla Hospital	Sputum	87	ICU	13	1	1	1	0	1	0	0	0	1	1	0	0	1	1	0
SK052	Songkhla Hospital	Abdomen	64	ICU	16	0	0	0	1	0	0	1	1	1	1	0	0	0	0	1
SK065	Songkhla Hospital	Sputum	33	ICU	12	0	1	0	0	1	0	0	0	1	1	0	1	0	0	0
SK068	Songkhla Hospital	Sputum	54	ICU	10	1	0	1	0	0	0	1	0	0	0	0	0	1	1	0

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*DM; diabetes mellitus, HTN; essential blood hypertension, CKD; chronic kidney disease, CVA; cerebrovascular disease, CAD; coronary heart disease, ICU; intensive care unit. MEM; meropenem ERT; ertapenem, LVX; Levofloxacin, CIP; ciprofloxacin, IMP; imipenem, CRO; ceftriaxone**,** PIP/TAZ; Piperacillin + tazobactam, CAZ; Cefazolin, AMG; aminoglycocides

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Educational Attainment

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List of Publication and Proceeding

Nwabor, L.C., Chukamnerd A., Nwabor, O. F., Pomwised R., Voravuthikunchai S.P and Chusri S. Rifampicin Enhanced Carbapenem Activity with Improved Antibacterial Effects and Eradicates Established *Acinetobacter baumannii* Biofilm. Pharmaceuticals 2023, 16(4), 477; https://doi.org/10.3390/ph16040477

Nwabor, L.C., Chukamnerd A., Nwabor, O. F., Surachat K., Pomwised R., Jeenkeawpiam K., Chusri S. Genotypic and phenotypic mechanisms

underlying antimicrobial resistance and synergistic efficacy of rifampicin-based combinations against carbapenem-resistant *Acinetobacter baumannii* (submitted).

Nwabor O F., Chukamnerd A., Terbtothakun Pawarisa., Nwabor L.C., Surachat K., Roytrakul S., Voravuthikunchai S.P, and Sarunyou Chusri. Synergistic effects of polymyxin and vancomycin combinations on carbapenem- and polymyxin-resistant *Klebsiella pneumoniae* and their molecular characteristics (submitted manuscript, microbiology spectrum).

Nwabor, L. C, Chukamnerd, A. and Sarunyou C. Rifampicin and Colistin Combination: A Potential Therapy for the Treatment against Carbapenem-Resistant *Acinetobacter Baumannii* Infections. International Conference on infectious Diseases Medicine, Infectious Diseases and Immunotherapy.5th April 2023, Phuket, Thailand (ITAR-ICIDMIDI-23 page 17).